Six2 EXHIBITS A TEMPORAL-SPATIAL EXPRESSION PROFILE IN THE DEVELOPING MOUSE PALATE AND IMPACTS CELL PROLIFERATION DURING MURINE PALATOGENESIS

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfilment of the Requirements for the Degree of Master of Science in the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon

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ABSTRACT

Cleft palate is one of the most common congenital malformations in humans which occurs at a frequency of approximately 1:700 live births worldwide. *Sine Oculis-related homeobox 2* (*Six2)* is a member of the vertebrate *Six* gene family that encode proteins that are transcription factors. *Six2* has been reported to be a downstream target of *Homeobox a2* (*Hoxa2)*, a gene that plays a direct a role in mouse secondary palate (SP) development.

In my thesis, I utilized quantitative real time Polymerase Chain Reaction (qPCR), Western blot analysis and fluorescence immunohistochemisrty (IHC) to characterize the spatial and temporal distribution patterns of *Six2* in the developing SP. Additionally, I also employed *in vivo* cell counting analysis and *in vitro* cell proliferation assays to investigate the role of *Six2* during palate mesenchymal cell proliferation.

My study examined the temporal and spatial distribution of *Six2* in the developing mouse palatal mesenchyme and epithelia in both wild-type and *Hoxa2* null mice. *Six2* was expressed throughout the period of embryonic palatogenesis, with the highest levels of *Six2* mRNA and protein observed in palatal shelves at E13.5 in both wild-type and *Hoxa2* null mice. Six2 protein expression at all stages of SP development (E12.5 to E15.5) increased in the anterior to posterior (A-P) direction with highest expression in the posterior regions of the developing SP. In addition, expression of Six2 protein was higher in the oral half of the palatal mesenchyme compared to the nasal half of the palatal mesenchyme. Interestingly, Six2 protein was expressed in the nasal palatal epithelium but was completely absent from the oral palatal epithelium. Loss of the *Hoxa2* gene induced up regulation of Six2 protein and mRNA in the developing palate across all stages of palatogenesis. In the *Hoxa2* null mice, there was a significant increase in cell proliferation (Ki-67 positive cells) and

the percentage of actively proliferating cells that were co-expressing Six2 protein (Six2/Ki-67 double positive cells) along both the A-P and oral-nasal (O-N) axes of the developing SP. Also, the highest percentage of actively proliferating cells and *Six2/Ki-67* double positive cells was observed in the nasal half of the posterior palatal mesenchyme. Furthermore, *Six2* siRNA knock down in mouse embryonic palatal mesenchyme (MEPM) cell cultures restored cell proliferation and *Cyclin D1* expression in the *Hoxa2* null cell cultures to wild-type levels.

Collectively, my data reveals a novel spatial and temporal expression profile for *Six2* in the developing mouse SP and the potential role it might play during the epithelial-mesenchymal cross talk that drives palatal shelf cell proliferation and out growth.

ACKNOWLEDGEMENTS

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Finally, I would like to specially thank the College of Pharmacy and Nutrition of the University of Saskatchewan for funding part of my studies.

DEDICATION

This Degree is dedicated to my supportive family especially my Dad, Dr. John Okorio, without whom I would never have dreamt of coming to Canada to pursue graduate studies. I will forever be indebted to you for your support both financially and morally, that you have rendered me in my never-ending pursuit for knowledge. Thank you for believing in me.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
A-P	Anterior-Posterior
BA	Branchial Arch
Barx1	BarH-like homeobox 1
Bmp4	Bone morphogenetic protein 4
bp	base pair
C	Celsius
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
CL/P	Cleft lip and palate
CNCC	Cranial neural crest cell
CO_2	Carbon dioxide
CP	Cleft Palate
Ct	Threshold cycle
ddPCR	Droplet digital Polymerase Chain Reaction
Dlx5	Distal-less homeobox 5
DM	Myotonic dystrophy
DMEM	Dulbecco's modified eagle's medium
DMPK	Myotonic dystrophy protein kinase
DMWD	Myotonic dystrophy with beta-transducin (WD) repeat
DNA	Deoxyribonucleic acid
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
Eya1	Eyes absent1
Fgf	Fibroblast growth factor
Fgf10	Fibroblast growth factor 10
Fgfr	Fibroblast growth factor receptor
FIG	Figure
Foxf1a	Forkhead box F1a
Foxf2	Forkhead box F2
h	Hour
HD	Homeodomain
Hoxa2	Homeobox a2
HPE	Holoprosencephaly
IDV	Integrated density value
IgG	Immunoglobulin G
IHC	immunohistochemistry/immunohistochemical
Italics	Denotes the gene or mRNA form

M	Molar
Meox2	Mesenchyme homeobox 2
MEPM	Mouse embryonic palatal mesenchyme
MES	Midline Epithelial Seam
mg	milligrams
min	Minutes
mM	Millimolar
Mn1	Meningioma 1
MNP	Medial nasal prominence
mRNA	Messenger Ribonucleic acid
Msx1	msh-like1
MXP	Maxillary prominence
n	Number of samples
NaCl	Sodium chloride
NCC	Neural crest cell
ng	Nanogram
nM	Nanomolar
^{o}C	Degrees centigrade
OCT	Optimal cutting temperature
O-N	Oral-Nasal
Osr1	Odd-skipped related 1
Osr2	Odd-skipped related 2
Pax9	Paired box gene 9
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PP	Primary Palate
Ptc	Patched 1
Ptx1	Paired like homeodomain transcription factor1
PVDF	Polyvinylidene difluoride
RIPA	Radio immuno precipitation assay buffer
RQ	Relative quantification
RT	Reverse transcription
RTM	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s	Seconds
SD	*Six* domain
Shh	Sonic hedgehog
Shox2	Short stature homeobox 2
siRNA	Small interfering ribonucleic acid
Six	Sine Oculis-related homeobox

Six2	Sine Oculis-related homeobox 2
Smo	Smoothened
SM-PBS	Skim milk in phosphate buffered saline
SM-PBST	Skim milk in phosphate buffered saline with 1% Triton X-100
SP	Secondary palate
Spry2	Sprouty homolog 2
Tbx22	T-box transcription factor 22
Tgfβ	Transforming growth factor beta
TGif	TG-interacting factor
UTR	Untranslated region
UV	Ultraviolet
Wt	Wild-type
µl	Microlitre
µm	Micrometer
µM	Micromolar

1. INTRODUCTION

Cleft palate is one of the most common congenital malformations in humans, occuring at a frequency of approximately 1:700 live births worldwide (Gorlin et al., 2001). The palate separates the nasal and oral cavities and this allows for respiration, feeding and phonation to occur. Environmental and genetic factors have both been implicated in the pathogenesis of the cleft palate. Maternal use of the anticonvulsant drugs valproic acid and phenytoin, has been shown to cause cleft palate in the newborn (Holmes et al., 2001). However, the molecular mechanisms involved in this drug-induced manifestation are poorly understood.

Craniofacial development is a tightly regulated process that involves the growth and fusion of the maxillary, mandibular and nasal processes (Chai and Maxson, 2006). Mouse secondary palate development begins around E11.5. At E12.0-13.5, paired palatal shelves initiate from the oral side of the maxillary processes and grow vertically downwards on either side of the tongue. The tongue depresses at E14.0 causing the palatal shelves to reorient above the tongue. These then grow horizontally towards each other ultimately touching at E14.5 to form the midline epithelial seam (MES). The MES degrades by E15.5, leaving behind a confluent secondary palate. In addition, the secondary palate fuses anteriorly with the primary palate, a derivative of the medial nasal processes, to form the intact roof of the oral cavity. Therefore, disruption of the growth, elevation or fusion of the palatal shelves can lead to cleft palate (Bush and Jiang, 2012; Ferguson, 1988; Gritli-Linde, 2007, Smith et al., 2013).

Six2 is a member of the vertebrate gene family consisting of the following genes: *Six1, Six3, Six4, Six5* and *Six6* which encode transcription factor proteins homologous to the *Drosophila* 'sine oculis' homeobox protein (Kiyoshi et al., 2000). *Six2* has been shown to be involved in regulating

craniofacial and kidney development (Fogelgren et al., 2008, 2009; Lozanoff, 1993; Lozanoff et al., 2001; Ma and Lozanoff, 1996, 1999; McBratney et al., 2003; Singh et al., 1998). *Six2* expression has been reported in the mesenchyme of the nasal prominences of E11.5 mice (Brodbeck et al., 2004; Fogelgren et al., 2008; Ohto et al., 1998; Oliver et al., 1995). *Six*2 has also been reported to be a direct downstream target of *Hoxa*2 in the murine branchial arches (Kutejova et al., 2005; Kutejova et al., 2008).

Although *Six2* has been reported to be expressed during mammalian craniofacial development, no detailed expression analysis has been performed in the developing secondary palate. In addition, its precise role in the craniofacial region and especially in the developing mammalian secondary palate is still unknown. My thesis therefore seeks to address these gaps in knowledge regarding the transcription factor *Six2* during palatogenesis.

2. REVIEW OF LITERATURE

2.1 An overview of craniofacial development

2.1.1 Early embryonic development

In the human embryo, oral development starts early by the appearance of the prechordal plate in the bilaminar germ disc on the 14th day of development. The endodermal thickening of the prechordal plate demarcates the cranial pole of the oval embryonic disk, which later contributes to the oropharyngeal membrane. This bilaminar membrane is the site of the junction of the ectoderm that forms the mucosa of the mouth and the endoderm that forms the mucosa of the pharynx, which is the most cranial part of the foregut. The oropharyngeal membrane is one of two sites of contact between ectoderm and endoderm, where mesoderm fails to intervene between the two primary germ layers; the other site is the cloacal membrane at the terminal end of the hindgut. The oropharyngeal membrane also demarcates the stomodeum, the primitive mouth that forms the center of the developing face (Richman et al., 1995; Sulik et al., 1990).

The development of the head relies on the inductive activities of the prosencephalic and rhombencephalic organizing centres that are regulated by *sonic hedgehog* (*Shh*) (Hu et al., 1999) (Fig. 1). Located at the rostral end of the notochord beneath the forebrain (prosencephalon) is the prosencephalic centre which is derived from the prechordal mesoderm that migrates from the primitive streak. This centre induces the visual and inner-ear apparatus and the upper third of the face through Shh protein signalling. Similar to the prosencephalic centre, the caudal rhombencephalic centre located beneath the hindbrain (Rhombencephalon) also induces the middle and lower thirds of the face including the middle and external ears through *Shh* signalling. In

association with these developments is the bifurcation of the unilobar forebrain (prosencephalon) to form paired telencephalic hemispheres and evagination of the paired olfactory bulbs and optic nerves. Failure of these cerebral divisions leads to severe facial anomalies (Johnson et al., 1995).

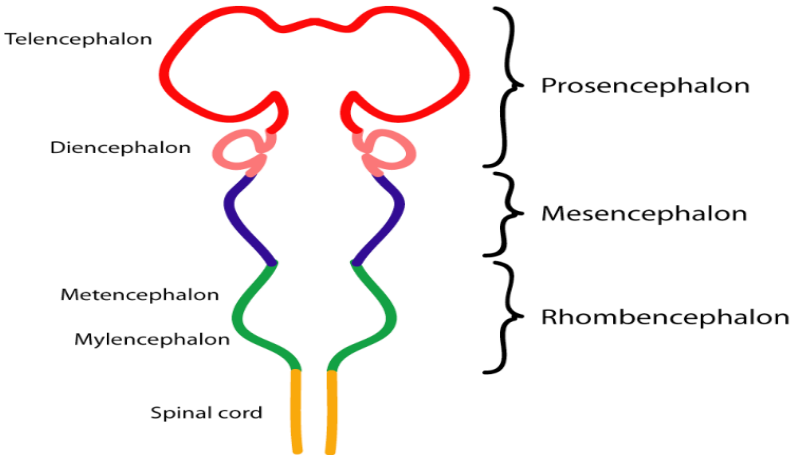


Figure 1: A schematic illustration of showing the three primary brain vesicles of an early embryo (prosencephalon, mesencephalon and rhombencephalon) and their five main subdivisions.
(http://what-when-how.com/neuroscience/development-of-the-nervous-system-gross-anatomy-of-the-brain-part-1/. Web. 0ctober 4th 2014).

2.1.2 Migration of the cranial neural crest cell population

Formation of cranial neural crest cells (CNCC) is one of the key features of craniofacial development. The specification, emigration and migration, proliferation, survival and ultimate fate determination of the CNCC play an important role in regulating craniofacial development. CNCC give rise to a variety of cell types during embryonic development. These cells eventually give rise to the hard tissues of the head such as bone, cartilage and teeth; optic elements such as the choroid, sclera, iris, and ciliary body, and sensory ganglia of the fifth, seventh, ninth and tenth cranial nerves (Noden, 1983; Couly et al., 1993; Kontges and Lumsden, 1996) (Fig. 2). Genetic disorders, environmental insults, or a combination of both can alter the fate determination of CNCC leading to craniofacial malformations.

CNCC arise uniformly at the dorso-lateral edge of the closing neural folds, along almost the entire length of the vertebrate embryo neural axis. This region corresponds to the interface between the non-neural ectoderm (presumptive epidermis or surface ectoderm) and the neural plate (neuroepithelium), a region that is commonly referred to as the neural plate border. CNCC induction requires contact mediated interactions between the surface ectoderm and neuroepithelium and each of these tissues contributes to the neural crest cell lineage (Selleck and Bronner-Fraser, 1995). In addition to their induction along the dorsolateral edge of the neural plate, neural crest cells undergo an epithelial to mesenchymal transformation whereby they delaminate from the neural tube and begin to migrate (Figs. 2, 3). These epithelial-mesenchymal transitions are marked by changes in cell adhesion and cytoarchitecture (Bronner-Fraser, 1993; LaBonne and Bronner-Fraser, 1999; Le Douarin et al., 2004). Following delamination from the neural tube, CNCC migrate along specific pathways to their final destinations (Fig. 4). In

vertebrates, the majority of CNCC are derived from the hindbrain, which migrate ventrolaterally from neural tube in three distinct sub-ectodermal streams adjacent to the even numbered rhombomeres (r2, r4, r6). The proliferative activity of the CNCC in the craniofacial region eventually leads to the formation of the frontonasal processes and discrete swellings that demarcate each branchial arch. The three streams of CNCC populate the first, second and third branchial arches respectively in keeping with their craniocaudal axial origins (Lumsden et al., 1991) and give rise to a wide variety of cell lineages, which are distinct for each branchial arch (Noden, 1983; Kontges and Lumsden, 1996).

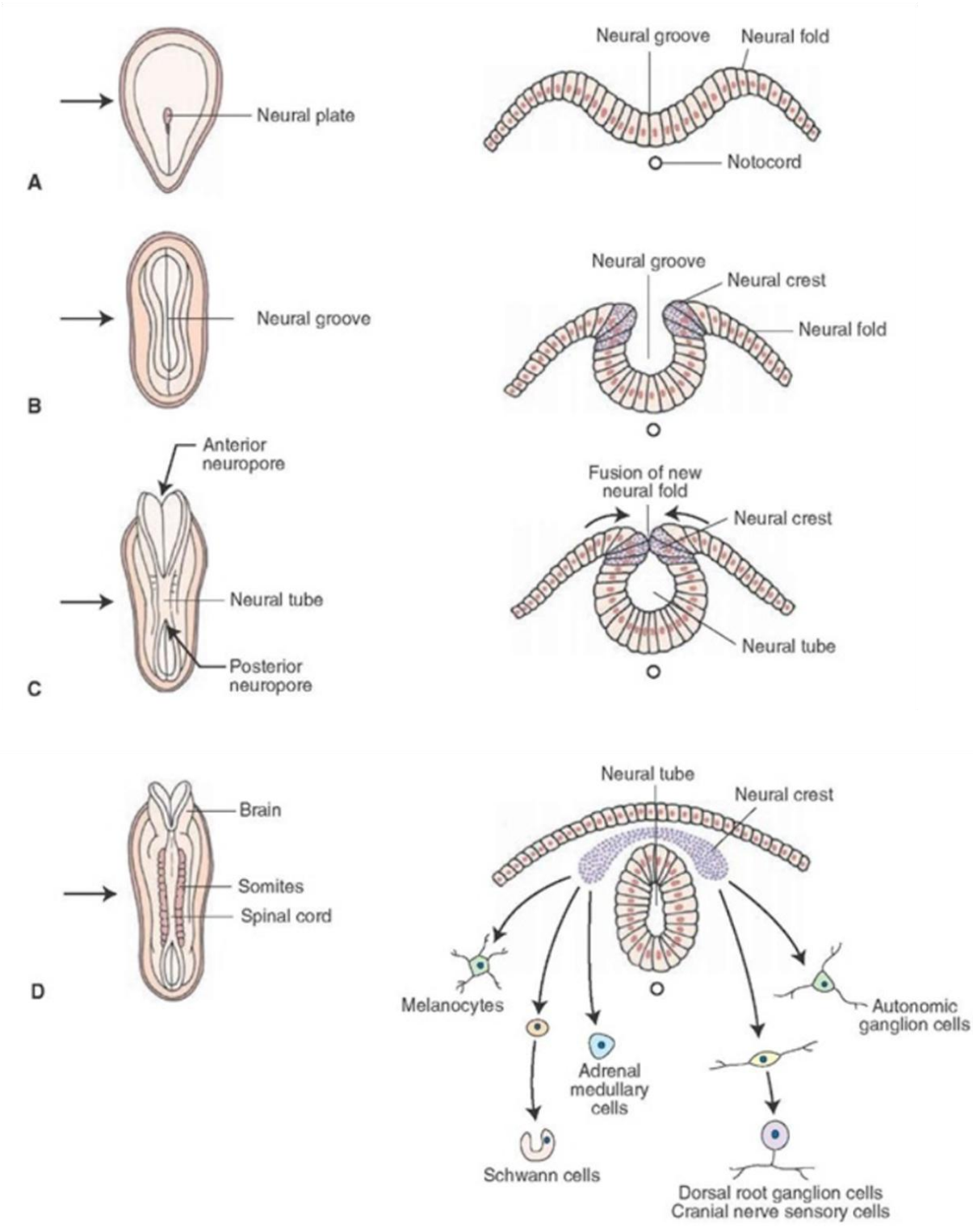

A
Neural plate
Neural groove
Neural fold
Notocord
B
Neural groove
Neural groove
Neural crest
Neural fold
C
Anterior neuropore
Neural tube
Posterior neuropore
Fusion of new neural fold
Neural crest
Neural tube
D
Brain
Somites
Spinal cord
Neural tube
Neural crest
Melanocytes
Autonomic ganglion cells
Adrenal medullary cells
Schwann cells
Dorsal root ganglion cells
Cranial nerve sensory cells

Figure 2: A schematic representation of early embryonic development. Panels A-D depict early development (at the third and fourth weeks of gestation) in which the neural plate (A), neural groove (B), and neural tube (C) are formed from the dorsal surface of the embryo. The left side of each panel depicts the developing embryo in a dorsal view, and the right side shows cross sections through the nervous system cut at the levels indicated by the arrows. Note also the cells formed from differentiated cells of the neural crest (D).
(Taken from http://what-when how.com/neuroscience/development-of-the-nervous-system-gross-anatomy-of-the-brain-part-1/. Web. October 10th 2014).

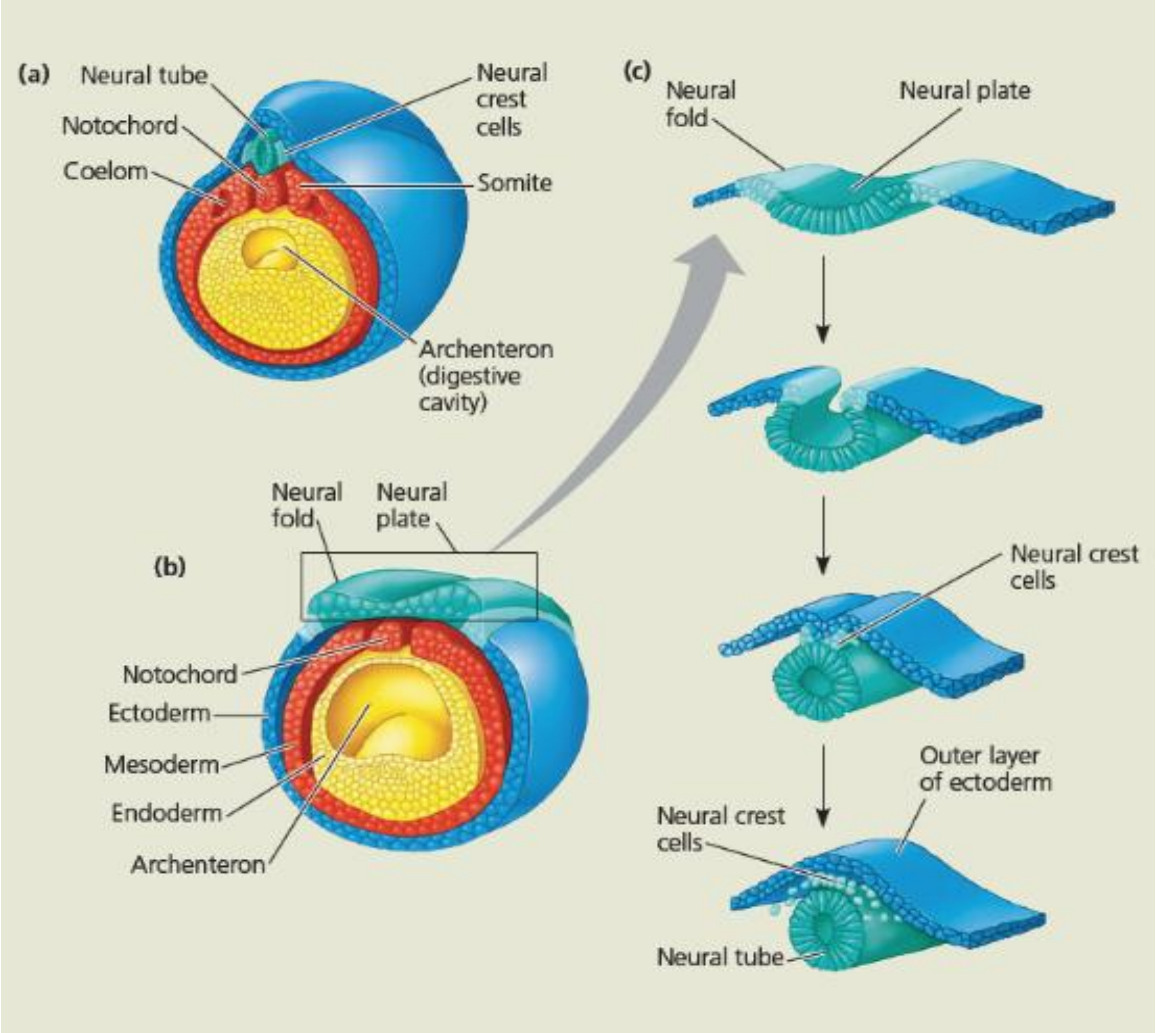


Figure 3: Diagrammatic illustration of cranial neural crest cell formation and delamination.

(REECE, JANE B.; URRY, LISA A.; CAIN, MICHAEL L.; WASSERMAN, STEVEN A.; MINORSKY, PETER V.; JACKSON, ROBERT B., CAMPBELL BIOLOGY, 9th Edition, © 2011. Reprinted and Electronically reproduced by permission of Pearson Education, Inc., Upper Saddle River, NJ).

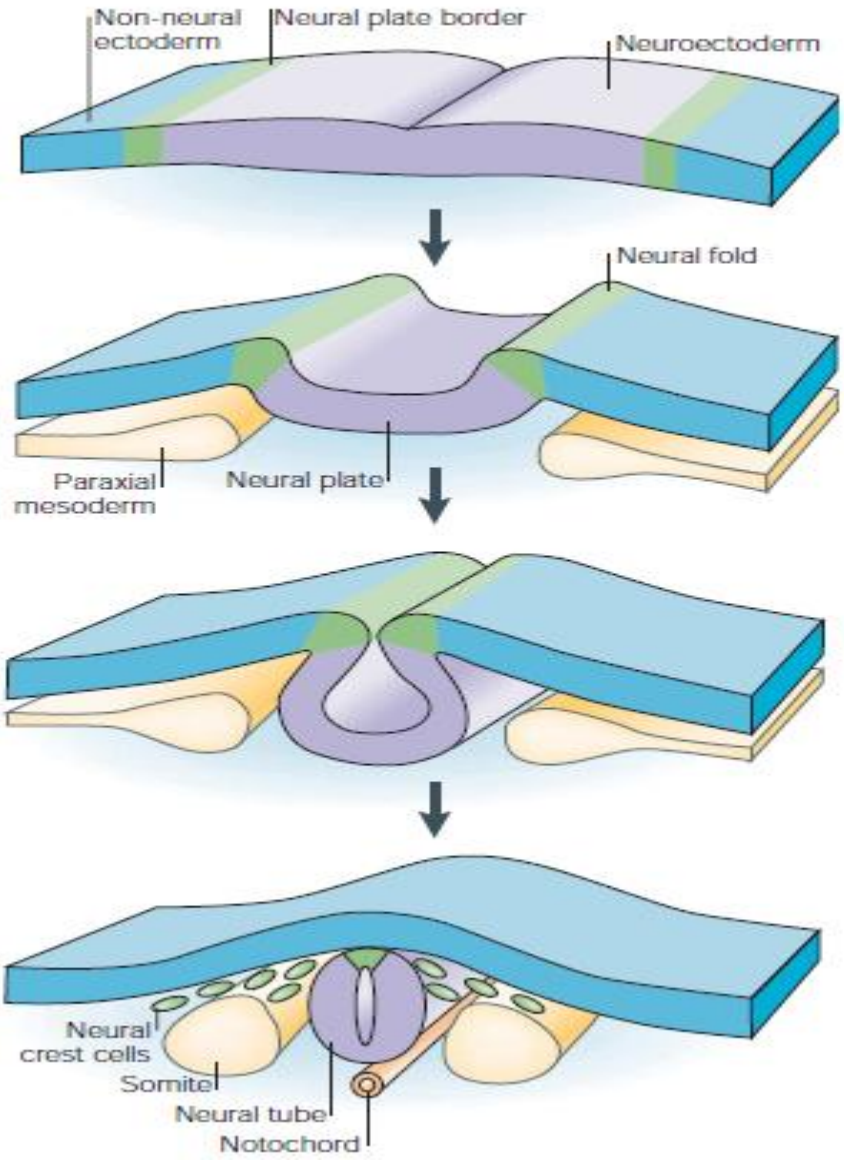


Figure 4: A schematic demonstration of border induction and neurulation. The neural plate border (green) is induced by signalling between the neuroectoderm (purple) and the non-neural ectoderm (blue) and from the underlying paraxial mesoderm (yellow). During neurulation, the neural plate borders (neural folds) elevate, causing the neural plate to roll into a neural tube. Neural crest cells (green) delaminate from the neural folds or the dorsal neural tube (shown), depending on the species and axial level.
(Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience., Neuro crest specification: migrating into genomics., copyright Oct 1, 2003.)

2.1.3 Formation of the face

The face originates from five prominences that surround a central depression, the stomodeum, which constitutes the future mouth. These prominences include the single median frontonasal and the paired maxillary and mandibular prominences ; the latter two are derivatives of the first pair of six branchial arches. These prominences and arches arise in most part, from neural crest ectomesenchyme (neural crest cells) that migrates from the dorsal neural tube (point of fusion of the neural plate borders) into the facial and neck regions (Fig. 2).

The frontonasal prominence surrounds the forebrain, which has lateral optic diverticula that form the eyes. The frontal portion of the prominence between the eyes forms the forehead; at the inferolateral corners, thickened ectodermal nasal (olfactory) placodes arise. These placodes become the olfactory epithelium and develop the underlying olfactory nerves. Union of the facial prominences occurs by merging of the frontonasal, maxillary, and mandibular prominences. Merging of what are initially incompletely separated prominences occurs as the intervening grooves disappear as a result of migration into and proliferation of underlying mesenchyme in the groove. Fusion of the freely projecting medial nasal prominences with the maxillary and lateral nasal prominences on each side requires the disintegration of their contacting surface epithelia, allowing the underlying mesenchymal cells to intermingle. Failure of normal disintegration by cell death or mesenchymal transformation is a cause of cleft upper lip and primary palate, as such failure prevents the intermingling of maxillary and medial nasal mesenchyme. Fusion of the medial nasal and maxillary prominences provides for continuity of the upper jaw and lip and separation of the nasal pits from the stomodeum. The midline merging of the medial nasal prominences forms the median

tuberculum and philtrum of the upper lip, the tip of the nose, and the primary palate.

The intermaxillary segment of the upper jaw (the premaxilla), in which the four upper incisor teeth will develop, arises from the median primary palate that is initially a widely separated pair of inwardly projecting swellings of the merged medial nasal prominences. Abnormal unilateral clefting resulting from failed fusion of medial nasal and maxillary prominences produces a deflection of the nose and upper lip.

The lower jaw and lip are very simply formed by midline merging of the paired mandibular prominences and are the first parts of the face to become definitively established. The lateral merging of the maxillary and mandibular prominences creates the commissures (corners) of the mouth (Fig. 5) (Gilbert, 2003; Lumsden and Keynes, 1989; Helms et al., 2005; Sperber and Sperber, 2009; Hinrichsen, 1985).

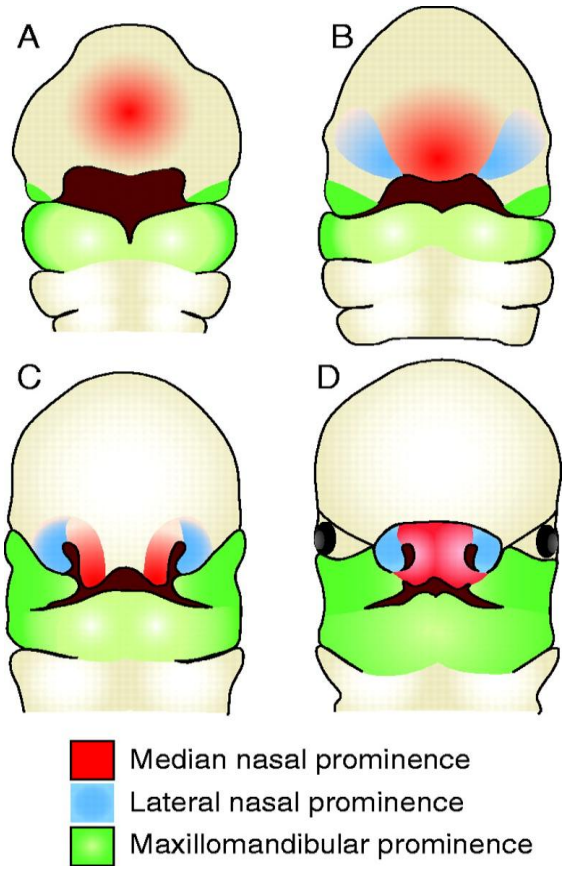


Figure 5: **A diagrammatic illustration of facial development Showing the prominences from which the structures of the face are formed.** The frontonasal or median nasal prominence (red) gives rise to the forehead (A), centre of the nose (B), philtrum of the upper lip (C) and the primary palate (D). The lateral nasal prominences (blue) form the sides of the nose (B,D). The maxillary and mandibular prominences (green) give rise to the sides of the middle and lower face, lateral borders of the lips, the secondary palate (specifically from maxillary prominences) and to the lower jaw (specifically from the mandibular prominences) (D).
(REPRODUCED WITH PERMISSION FROM HELMS JA, CORDERO D, TAPADIA MD. (2005). NEW INSIGHTS INTO CRANIOFACIAL MORPHOGENESIS. DEVELOPMENT 132:851–861).

2.2 Secondary palate development

The primary and secondary palates form the main barrier between mammalian nasal and oral cavities. This was an evolutionary advancement from the single common chamber seen in birds and reptiles (Chong et al., 2002 and Sperber et al., 2002). The secondary palate (SP) is the primordium of both the hard and soft palates of the palate. SP development is a multi-step process involving palatal shelf growth, elevation, midline fusion of the two palatal shelves and the disappearance of the midline epithelial seam (MES). The palatal structures are composed of the cranial neural crest (CNC-derived ectomesenchyme and pharyngeal ectoderm) (Ferguson, 1988; Shuler, 1995).

Palatal shelves are covered by epithelia that can be divided into the nasal, oral and medial edge epithelium (MEE). The nasal and oral epithelia differentiate into pseudostratified columnar and stratified squamous epithelia respectively, whereas the medial edge epithelium gets removed by means of programmed cell death (apoptosis) and cell migration (Martinez-Alvarez et al., 2000; Vaziri Sani et al., 2005).

Secondary palate development begins at E11.5 in mice and around the sixth week of gestation in humans. Initially, the palatal shelves exist as projections in the internal aspects of the maxillae, on either side of the tongue. These two shelves then grow vertically downwards on either sides of the tongue between the stages E12.0 and E13.5 (Ferguson, 1988; Berkovitz,et al., 2002). As the jaws develop, the tongue depresses causing the palatal shelves to re-orient themselves above the tongue at E14.0. The shelves then start growing horizontally towards each other where they ultimately touch at E14.5 to form the midline epithelial seam (MES). The MES degrades by E15.5 leaving behind a confluent SP (Fig. 6).

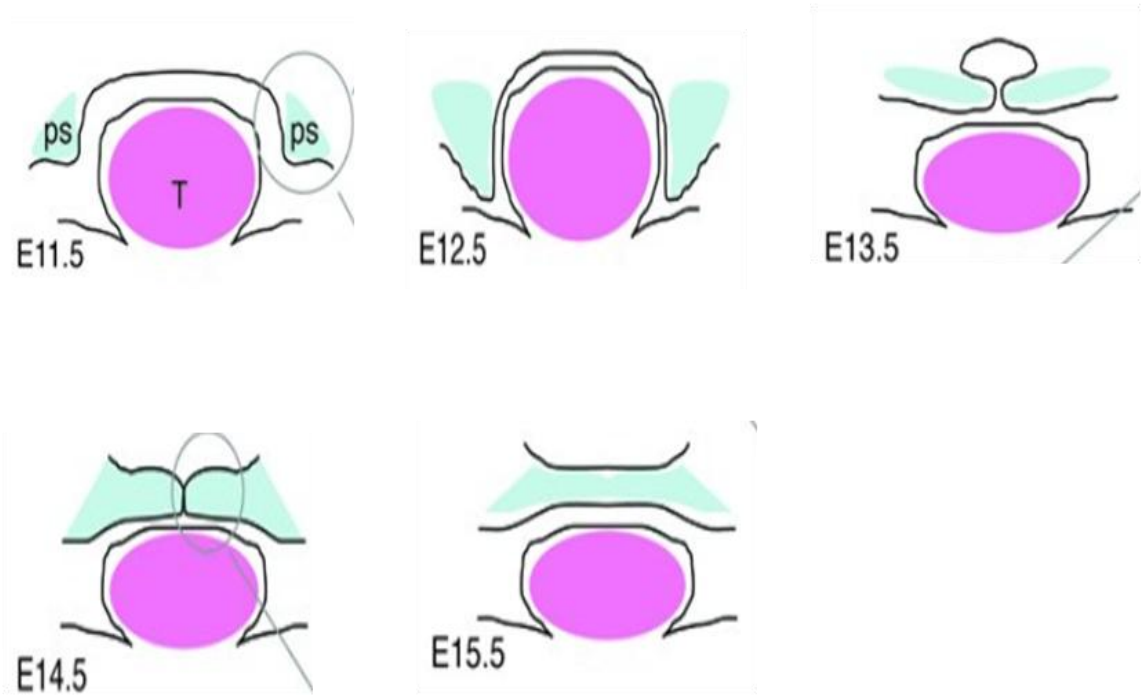


Figure 6: A schematic illustration of secondary palate development showing the palatal shelves emerging from the maxillary prominences at E11.5, growing vertically downwards on either side of the tongue (E12.5), re-orienting above the tongue (E13.5-E14.0), growing horizontally and touching to form the midline epithelial seam (E14.5) and eventually fusing to form a solid, confluent secondary palate (E15.5). ps- Palatal shelf, T-tongue.
(REPRODUCED WITH PERMISSION OF THE AMERICAN SOCIETY OF CLINICAL INVESTIGATION IN THE FORMAT REPUBLISH IN A THESIS/DISSERTATION VIA COPYRIGHT CLEARANCE CENTRE).

2.3 Molecular signalling in the developing palate

Mammalian SP development is an intricate process involving the participation of several transcription factors and growth factors that interact with their receptors as well as with each other. These networks work together to regulate critical cellular processes including cell proliferation, migration and epithelial-mesenchymal transdifferentiation and apoptosis. Similar to the development of other organs in the embryo, SP palate development relies on the sequential and reciprocal epithelial-mesenchymal interactions. Some of the transcription factors and growth factors involved in SP development include; *Shh, Msx1, Bmp4, Barx1, Fgf10, Fgf* receptors, *Pax9, Osr2* and *Tgfβ*.

Shh is a member of the hedgehog family of secreted proteins which plays crucial roles in diverse developmental processes including left-right axis establishment, dorso-ventral patterning of the neural tube, endoderm development, A-P patterning of the developing limb, brain development and patterning (McMahon et al., 2003; Roessler and Muenke, 2003). *Shh* is expressed at all stages of SP development and follows a striped pattern of expression corresponding to areas of thickened epithelium that later form the palatal rugae. The A-P outgrowth of palatal shelves has been associated with periodic formation of palatal rugae and is controlled by reciprocal epithelial-mesenchymal crosstalk along the A-P axis (Lan and Jiang, 2009; Pantalacci et al., 2008; Welsh and O'Brien, 2009). Palatal rugae have been reported to express high levels of *Shh* in addition to acting as signalling centers involved in epithelial-mesenchymal interactions required to coordinate palate outgrowth and patterning (Lan and Jiang, 2009; Rice et al., 2006; Welsh and O'Brien, 2009). A study by Han et al., 2009 reported that the epithelial cells expressing *Shh* are not actively proliferating, but the mesenchymal cells underlying these regions are more highly proliferative than mesenchymal cells in other areas of

the palate. *Shh* is a downstream target of the *Msx1* network that regulates cell proliferation in the anterior palate (Zhang et al., 2002).

Loss of the *Spry2* gene has been associated with the disorganization in the expression pattern of *Shh*, which ultimately leads to deformities in the rugae in the palate of these knockout animals (Welsh et al., 2007). Studies of mice involving epithelium-specific inactivation of *Shh* or mesenchyme-specific inactivation of smoothened (*Smo*), which encodes a transmembrane protein required for transducing *Shh* signalling, have demonstrated that *Shh* signals from the epithelium to the underlying mesenchyme to promote palatal cell proliferation and outgrowth (Rice et al., 2004; Lan and Jiang, 2009). In addition, the exogenous application of *Shh* has been shown to induce a mitogenic response in palatal explant cultures (Zhang et al., 2002; Rice et al., 2004). This mitogenic effect is mediated, in part, by the cell cycle regulators *Cyclin D1* and *Cyclin D2*, the expression of which was reduced in the palatal mesenchyme of embryos with mesenchyme-specific inactivation of *Smo*.

Lan and Jiang, (2009) reported that *Shh* from palatal epithelial cells stimulates cell proliferation and the expression of *Fgf10* in the developing palatal mesenchyme. *Shh* and *Fgf10* have also been reported to function in a positive-feedback loop to drive the outgrowth of the palatal shelves (Bush and Jiang., 2012; Lan and Jiang., 2009; Rice et al., 2004). The expression of *Foxf1a* and *Foxf2* and *Osr2* genes was also reduced in the palatal mesenchyme, indicating that these transcription factors might be downstream effectors of *Shh* signalling (Lan and Jiang, 2009). *Fgf10* is a crucial mesenchymal signal that is required for palatal outgrowth. Mice homozygous for a null mutation in either *Fgf10* or the gene encoding its receptor, *Fgfr2b*, exhibited cleft palate with impaired palatal shelf outgrowth (Rice et al., 2004). Whereas *Fgf10* mRNA expression was restricted to the mesenchyme, *Fgfr2b* mRNA was most

abundantly detected in the overlying epithelium. *Fgfr2* function is required within the epithelium, as mice harbouring an epithelial-specific deletion of *Fgfr2* also exhibited cleft palate (Hosokawa et al., 2009). Both epithelial and mesenchymal cell proliferation were reduced in the absence of either *Fgf10* or *Fgfr2b*, however, suggesting the presence of a factor that signals from the epithelium back to the underlying mesenchyme and that is dependent on *Fgf10/Fgfr2b* signalling. Indeed, *Shh* expression was dramatically reduced in the epithelium of *Fgf10* null and *Fgfr2b* null embryos, implying that the decreased palatal mesenchymal cell proliferation observed in these mutants might be a consequence of reduced *Shh* expression in the epithelium (Rice et al., 2004). The expression of *Fgf10* was also reduced in the palatal mesenchyme of embryos lacking mesenchymal *Smo*, indicating that *Shh* and *Fgf10* function in a positive-feedback loop that drives the outgrowth of the palatal shelves (Lan and Jiang, 2009).

In addition, cross-regulation of the *Shh* and *Bmp* signalling pathways has also been detected in the developing palate (Baek et al., 2011; Lan and Jiang, 2009; Zhang et al., 2002). The loss of *Smo* within the palatal mesenchyme led to up regulation of *Bmp4* and down regulation of *Bmp2* (Lan and Jiang, 2009). The positive regulation of Bmp2 by *Shh* signalling is consistent with the finding that exogenous *Shh*-containing beads induce *Bmp2* expression in palatal explant culture (Zhang et al., 2002). Whereas complete inactivation of *Bmp4* resulted in early embryonic lethality, ablation of *Bmp4* function in the maxillary mesenchyme and throughout the oral epithelium caused a cleft lip phenotype, but no secondary palate defect was reported (Liu et al., 2005). Overexpression of the Bmp antagonist noggin specifically in the palatal mesenchyme led to retarded palatal growth and cleft palate in mice (Xiong et al., 2009), further supporting the involvement of *Bmp* signalling during palatal growth. Studies

have also shown that *Bmp* signalling during palatogenesis occurs via the type I Bmp receptor Bmpr1a. Disruption of *Bmpr1a* in the maxillary mesenchyme and throughout the oral epithelium resulted in cleft lip and palate (Liuet al., 2005).

Pax9 is a member of the transcription factor family characterized by the paired-class DNA binding domain (Stapleton et al., 1993). *Pax9*-deficient mice have been reported to die shortly after birth, exhibiting complete cleft palate (Peters et al., 1998). Yu et al., 2013 reported that *Pax9* regulates epithelial-mesenchymal signalling and A-P patterning during palate outgrowth and that *Pax9* acts upstream of *Osr2* to regulate palatal shelf growth and elevation. In addition, this study also reported that *Pax9* acts upstream of *Bmp4, Fgf10, Msx1* and *Osr2* in the palatal mesenchyme.

2.4 Molecular signalling along the A-P axis of the developing SP

Although the palatal shelves appear as continuous outgrowths that project from the maxillary processes into the oral cavity, studies have revealed differential gene expression along the A-P axis, and that this heterogeneity is evident from the early stages of palatal outgrowth (Hilliard et al., 2005; Li and Ding, 2007; Welsh and O'Brien, 2009; reviewed in Smith et al., 2013). The SP may be divided into three regions along the A-P axis, namely the anterior palate, medial palate and posterior palate. A number of different systems of nomenclature have been used to demarcate the boundaries of the anterior, medial and posterior regions of the SP. In this thesis, I will follow the nomenclature proposed by Smith et al., (2013) which defines the anterior and posterior palate as tissue lying anterior or posterior to the first molar tooth bud, respectively. The medial palate is defined as palate tissue in the plane of the molar tooth bud.

Several transcription factor genes are differentially expressed along the A-P axis of the developing palatal shelves. The development and maintenance of the expression of these genes in a region specific manner is critical for normal SP development. *Msx1*, *Bmp4, Bmp2, Shh, Spry2, Fgf10, Fgf7, Shox2* genes are preferentially expressed in the anterior palatal mesenchyme (Alappat et al., 2005; Rice et al., 2004; Yu et al., 2005; Zhang et al., 2002), whereas *Barx1*, *Mn1*, *Meox2*, *Tbx22* genes are preferentially expressed in the posterior palatal mesenchyme (Barlow et al., 1999; Li and Ding, 2007; Liu et al., 2008). Although mice lacking either *Msx1* or *Mn1* exhibited complete cleft palate, *Msx1* null mice exhibited specific cell proliferation defects in the anterior region, whereas *Mn1* null mice showed growth deficits in only the middle and posterior regions of the palatal shelves (Zhang et al., 2002; Liu et al., 2008). Mice lacking *Shox2* exhibited a cleft within the anterior palate, whereas the posterior palate fused normally, demonstrating a specific requirement for *Shox2* in anterior palatal outgrowth (Yu et al., 2005). *Tbx22* null mice displayed cleft palate, with the severity varying from a complete cleft palate phenotype due to decreased palatal shelf extension to submucous cleft palate (abnormal muscle orientation in the soft palate during midline fusion) in which palatal shelf elevation and fusion occurred normally (Pauws et al., 2009). The expression of *Msx1* and *Shox2* in the anterior palatal mesenchyme depends on *Bmp* signalling, as expression of both these genes was significantly reduced in the anterior palate of *Wnt1-Cre; Bmpr1af/–* mice (Li et al., 2011).

Most studies on palatal outgrowth have focused on the downward growth of the palatal shelves; however, the maxillary processes also undergo significant rostrocaudal expansion from E12.5 to E14.5, and the palatal shelves correspondingly elongate along their A-P axis. As the palatal shelves elongate,

the domain of *Shox2* mRNA expression expands much more dramatically than that of *Meox2* (Li and Ding, 2007). It has been demonstrated that the A-P boundary of *Shox2* and *Meox2* expression coincides with the first formed palatal rugae (Pantalacci et al., 2008; Welsh and O'Brien, 2009). The rugae are metameric epithelial thickenings on the oral surface of the palate that are first apparent at E12.0 and develop by periodic interposition as the palatal shelves elongate along the A-P axis (Pantalacci et al., 2008). Whereas the second and third rugae form sequentially and anteriorly to the first ruga, the fourth ruga forms between rugae 1 and 2, and, as the palatal shelves continue to elongate along the A-P axis, subsequent rugae formation only occurs between the newly formed ruga and ruga 1 (Pantalacci et al., 2008; Welsh and O'Brien, 2009). Depending on the strain background, mice develop a total of nine or ten rugae, which are maintained after birth.

2.5 Molecular signalling along the O-N axis of the developing SP

The developing palatal shelves also exhibit molecular heterogeneity along the O-N axis during vertical outgrowth with the lateral side forming the oral palatal mesenchyme and the medial side forming the nasal palatal mesenchyme following palatal shelf elevation. Palatal rugae begin to form on the oral side of the developing palatal shelves at E12.0, and the expression of *Shh* becomes restricted to the oral palatal epithelium. *Osr1* and *Osr2* exhibit graded expression in the developing palatal mesenchyme along the oral-nasal axis (Lan et al., 2004). At E13.5, *Osr1* mRNA expression is restricted to the oral side, whereas *Osr2* exhibits graded expression that is strongest in the oral mesenchyme and weaker in the nasal mesenchyme (Yu et al., 2013). Whereas *Pax9* is expressed throughout the O-N axis of the palatal mesenchyme in the posterior region, its expression has been reported to be lower in the oral half

than in the nasal half of the palatal mesenchyme in the anterior region (Yu et al., 2013). *Dlx5* has also been implicated in O-N patterning and palatal expansion. *Dlx5* is co-expressed with *Fgf7* in the oral half of the palatal mesenchyme, and *Fgf7* expression was found to be dramatically reduced in this domain in *Dlx5* null palatal shelves (Han et al., 2009). Although *Dlx5* null palatal shelves elevated and fused, the oral aspect of the palate was dramatically expanded and a malformed soft palate was observed (Han et al., 2009).

2.6 The *Six* family of genes

The *Six* gene family consists of vertebrate genes that have sequence similarities to the *Drosophila* homeobox gene, *Sine oculis* (*So*). These genes are characterized by the presence of a divergent DNA-binding homeodomain (HD, 60 amino acids) and an upstream region, the *Six* domain (SD, 110-115 amino acids), that could be involved in both determining DNA binding specificity and in mediating protein-protein interactions (Fig. 7). In mammals, several members of the family have been cloned and designated *Six1 to Six6* (Boucher et al., 1995,1996; Oliver et al., 1995; Gallardo et al., 1999; Granadino et al., 1999; Kawakami et al., 1996). The *Six* and homeodomain regions are highly conserved across the *Six* family, while other regions of these proteins are often conserved across vertebrate species, but not between family members. Mouse *Six1* and *Six2* exhibit a high degree of sequence similarity to each other (95% amino acid sequence identity over a region spanning the *Six* domain and homeodomains) and they also show a higher degree of sequence similarity to *Drosophila* Sine oculis (*So*) (87 and 95%, respectively, over the same region) than do other family members. *Six1* and *Six2* thus represent orthologues of *Sine oculis* (Oliver et al., 1995). Their expression patterns, however, differ from

that of *So*, which is primarily expressed in the developing visual system of the fly. In mice, fetal expression studies have demonstrated that neither *Six1* nor *Six2* is expressed in the developing eye, rather, there is notable expression in anterior neural structures, neural crest derived tissues, and head, trunk and limb mesenchyme, with *Six1* and *Six2* transcripts appearing to occupy complementary axial domains in the phalanges (Oliver et al., 1995; Ohto et al., 1998).

2.7 Expression patterns of *Six* genes

Six genes are diversely expressed during vertebrate embryogenesis and control the formation of various organs. In most cases, they positively regulate cell numbers within the developing embryo by promoting cell proliferation and survival and mainly act as transcription factors (Christensen et al., 2008; Kawakami et al., 2000).

In the mouse embryo, *Six3* and *Six6* are expressed in restricted areas: developing eye, brain and a few related sites. The expression patterns of these two mouse *Six* genes appear to share similar characteristics with *Drosophila Optix*, whose expression is detected only in the cephalic region of the embryo. In contrast, *Six1, Six2* and *Six4* show much broader expression. *Six1* and *Six4* show remarkably similar patterns of expression. They are expressed in two of the sensory organs in the head region (otic vesicles and nasal pits) and in the branchial arches. In addition, the expression of *Six1* and *Six4* extends posteriorly in dorsal root ganglia and somites which give rise to the skeletal muscle of the trunk and limbs. The expression in somites is similar to mesodermal expression of *So* and *D-Six4* in *Drosophila*. *Six2* is also expressed caudally in certain tissues, in addition to the rostral and cervical regions. For *Six5*, the expression pattern was studied by using transgenic mice carrying a

transgene containing its 4.3 kb promoter fragment fused to the *lacZ* reporter gene (Fig. 8). The mice showed expression of the reporter lacZ in neural, sensory, muscle, cartilage and other tissues (Heath et al., 1997).

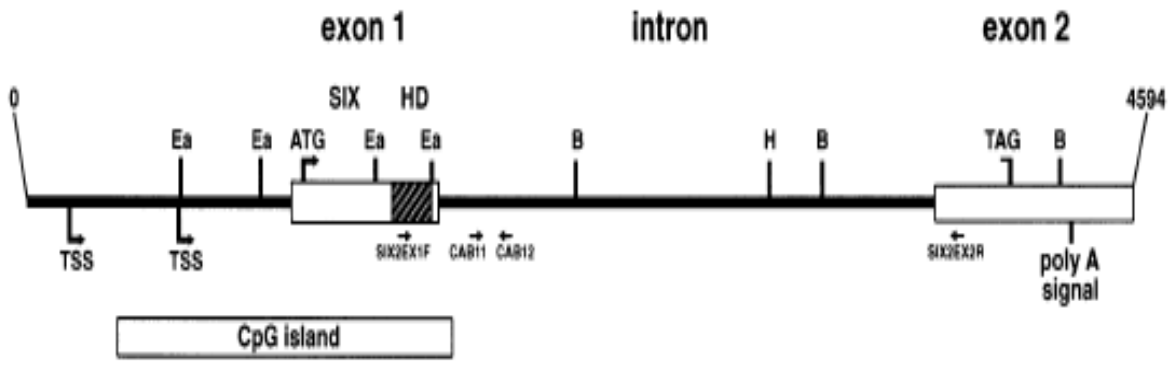


Figure 7: A schematic illustration of the human *SIX2* gene structure showing transcribed exonic sequences present in cloned cDNA (boxes) and the relative locations of the sequences encoding the SIX-(SIX) and homeodomains (HD). (Reproduced with permission from Boucher, C., Carey, N., Edwards, Y., Siciliano, M. and Johnson, K. (1996). Cloning of the human SIX1 gene and its assignment to chromosome 14. *Genomics* 33: 140-142., via Elsevier and copyright clearance centre).

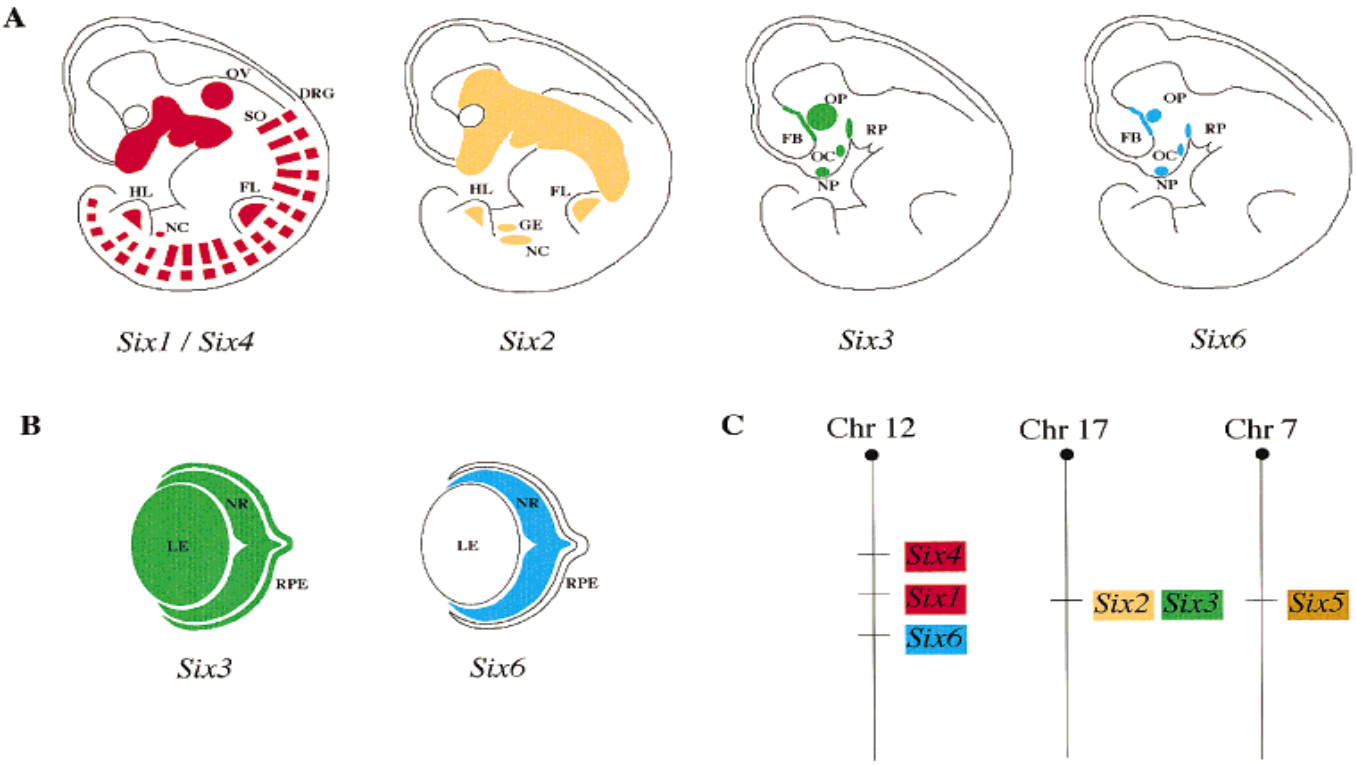


Figure 8: A schematic drawing of embryonic Expression patterns and chromosomal locations of mouse *Six* family genes. Similarity in the colour of shadings reflects similarity in the expression patterns. DRG, dorsal root ganglion; FB, forebrain; FL, forelimb; GE, genital eminence; HL, hindlimb; NC, nephrogenic cord; NP, nasal placode; OC, optic chiasm; OP, optic vesicle; OV, otic vesicle; RP, Rathke's pouch; SO, somites. B: Expression of Six3 and Six6 in a developing eye (E13.5). LE, lens; NR, neural retina; RPE, retinal pigmented epithelium. C: Chromosomal localization of mouse *Six* family genes.
Reproduced with permission from Kawakami, K., Sato, S., Ozaki, H. and Ikeda, K. (2000). *Six* family genes–structure and function as transcription factors and their roles in development. *BioEssays* 22: 616-626., via John Wiley and Sons and Copyright clearance centre.

2.8 *SIX* genes and genetic diseases

Holoprosencephaly (HPE) is a common malformation found in spontaneously aborted fetuses (1/250) and more rarely in live births (1/16,000). It is characterized by signs of abnormal development such as malformation of the brain, a single central incisor, hypotelorism, microcephaly and other craniofacial abnormalities (Roessler et al., 1998).

When the mouse *Six3* gene was mapped to the distal region of chromosome 17, (Oliver et al., 1995) which is orthologous to human 2p21 where the HPE2 locus resides, the possibility that the *SIX3* gene is involved in HPE was suggested. This idea was confirmed by the isolation of the *SIX3* gene from the HPE2 minimal critical region and the identification of four point mutations associated with HPE2 in the coding region of *SIX3.* All of those mutations were identified in the HD of *SIX3*; L226V and del(N232-S234) change amino acid residues unique to the *SIX3/6* subfamily while V250A and R257P change residues common to all Six proteins (Wallis et al., 1999). This strongly suggested that *SIX3* is the HPE2 gene essential for the development of the anterior neural plate and eye in humans.

Human *SIX6* haploinsufficiency was found to be responsible for bilateral anophthalmia, the absence of the optic nerve and chiasma, and for pituitary abnormalities (Gallardo et al., 1999). One such patient was found to have interstitial deletions of the 14q22.3-q23 chromosomal region. The regulatory region and the coding region of *SIX6* were both deleted in one chromosome 14 of that patient. The relationships between *SIX3/6* subfamily genes and several other genes implicated in HPE, such as *SHH*, (Belloni et al., 1996) Zic2,(Brown et al., 1998) and TG-interacting factor (*TGIF)* (Walsh et al., 1999) have not yet been clarified.

Myotonic dystrophy (DM) is characterized by muscle weakness, wasting and disturbances in the nervous system (Harris et al., 1996). In addition, DM is associated with ophthalmic features such as cataract, as well as cardiac conduction defects, mental impairment, premature balding and reduced fertility. *SIX5* was originally identified as DMAHP (DM associated homeodomain protein gene), which resides downstream of the causative CTG repeat located on 19q13.3 (DM1) (Boucher et al., 1995). Expansion of the CTG repeat results in increased severity of symptoms and an earlier age of onset. The CTG expansion at this locus has been proposed to alter the local chromatin structure and to repress the transcription of surrounding genes (Otten et al., 1995).

The expression of *SIX5* was reduced 2- to 4-fold in cells derived from DM1 patients relative to wild-type controls (Klesert et al., 1997; Thornton et al., 1997). At least some symptoms were believed to be caused by down-regulation of *SIX5*, which in turn leads to aberrant regulation of the *SIX5* target genes. For example, the observation that myogenin expression is up-regulated most efficiently by *Six5* and *Eya3* (one of the *Eya* family proteins in mouse) through the MEF3 site in the proximal promoter suggests the involvement of *SIX5* in muscle phenotype. The expression of *SIX5* but not DMPK in human lens also suggests the involvement of *SIX5* in the formation of cataracts (Winchester et al., 1999). The involvement of DMPK (DM protein kinase) and DMWD (DM protein with WD repeat), genes located 50 bp downstream to the CTG repeat, has also been suggested which would indicate that DM1 is a multi-gene disorder (Winchester et al., 1999; Alwazzan et al., 1999).

2.9 The *Six2* gene

Six2 is a homeobox transcription factor that is expressed in multiple organ systems of the developing embryo. This gene has been shown to be involved in regulating craniofacial and kidney development (Fogelgren et al., 2008; Fogelgren et al., 2009; Lozanoff, 1993; Lozanoff et al., 2001; Ma and Lozanoff, 1996; Ma and Lozanoff, 1999; McBratney et al., 2003; Singh et al., 1998). In addition, *Six2* has been reported to be expressed in the nasal prominences of E11.5 mice (Brodbeck et al., 2004; Fogelgren et al., 2008; Ohto et al., 1998; Oliver et al., 1995). *Six2* has been shown to be a downstream target of the Hox11 proteins in metanephric kidney development. In the early metanephric mesenchyme, Hox11 proteins interact with *Pax2* and *Eya1* to promote activation of *Six2* (Gong et al., 2007; Wellik et al., 2002). It has also been reported that *Six2* null mice die at birth and exhibit a shorter cranial base. In addition, these mice were also found to have defects in third and fourth branchial arch derived cartilages (Guiyuan et al., 2010). *Six2* has been reported to positively regulate cell numbers within the developing embryo by promoting cell proliferation and survival (Christensen et al., 2008; Kawakami et al., 2000). *Six2* has been implicated in kidney development where it maintains the renal progenitor cell population in an undifferentiated state (Self et al., 2006).

The epigenetic regulation of *Six2* by micro RNAs has also been reported. Lyu et al., (2013) reported that miR-181b directly targets the 3'UTR of *Six2* to downregulate its expression at both the mRNA and protein level in the kidney mesenchyme. In addition, Xiaoyan et al., (2014) have also reported that a single micro RNA (miRNA), miR181c was able to down regulate the expression of *Six2* in the kidney mesenchyme and thus restraining cell proliferation and promoting apoptosis. Furthermore, *Six2* has been reported to

promote breast cancer metastasis through the transcriptional and epigenetic control of *E-cadherin* expression (Wang et al., 2014).

Six2 is a direct downstream target of *Hoxa2*, a gene that plays a direct role in murine secondary palate development (Smith et al., 2009). Expression of *Six2* in wild type embryos is restricted to the first branchial arch mesenchyme. Loss of function of *Hoxa2* results in an expansion of *Six2* expression in the branchial arches and periodic mesenchyme and leads to malformations of the middle and external ear (Kujetova et al., 2005; Rijli et al., 1993). This implies that *Hoxa*2 expression is sufficient to repress the expression of *Six2* (Santagati et al., 2005). Interestingly, when *Six2* is overexpressed in the areas of the embryo controlled by *Hoxa*2, the result is a phenotype similar to the *Hoxa*2 mutant phenotype. Analysis of the promoter region of *Six*2 in combination with ChIP experiments suggests that *Hoxa*2 directly represses the expression of *Six*2 (Kutejova et al., 2005; Kutejova et al., 2008).

The studies above show the extent to which the *Six2* gene has been studied in mammalian development. Most of these studies have focussed on kidney development and craniofacial development in general. However, no detailed expression analysis of *Six2* has been performed in the developing secondary palate. In addition, its precise role in the craniofacial region and especially in the developing mammalian secondary palate is still unknown. My thesis therefore seeks to address these gaps in knowledge regarding the transcription factor *Six2* during murine palatogenesis.

3. HYPOTHESES

1. Development of the secondary palate is accompanied by changes in the temporal and spatial expression patterns of *Six2* gene transcripts and protein.
2. The *Six2* gene and protein are up-regulated in the absence of the *Hoxa2* gene in the secondary palate.
3. The *Six2* gene regulates cell proliferation during secondary palate development.

4. OBJECTIVES

1. To study the temporal and spatial expression pattern of the *Six2* gene and protein in the developing secondary palate.
2. To investigate whether *Six2* mRNA and protein are altered in the absence of the *Hoxa2* gene in the secondary palate
3. To determine if the *Six2* gene regulates cell proliferation during secondary palate development.

5. MATERIALS AND METHODS

5.1 Hoxa2 transgenic mice

Hoxa2 $^{+/+}$, *Hoxa2*$^{+/-}$ and *Hoxa2* $^{-/-}$ C57 black mice (Gendron-Maguire et al., 1993) were obtained by timed heterozygous matings. Heterozygous animals were obtained by backcrossing heterozygous males with wild-type females. After pups were weaned, animals were anaesthesized using isoflurane 3% in oxygen and approximately 0.2 cm piece of the tail was docked. Genotypes were confirmed by polymerase chain reaction (PCR) analyses (Appendix Fig. 2). Timed heterozygous matings were then performed, and were staged according to Kaufman with mice being considered E0 days pregnant on the day the vaginal plug was found (Kaufman, 1992).

5.2 PCR analysis of genotype

Tissue sample (tail or embryonic tissue) was digested overnight at 55°C in a solution containing 0.5M EDTA, Nuclei lysis solution (Promega) and 20 mg/ml proteinase K enzyme. Following the digestion, samples were allowed to cool to RTM after which protein precipitation solution (Promega) was added and the solution vortexed vigorously at high speed for 20 sec. Solution was then chilled on ice for 5 min followed by centrifugation for 4 min at 14,000 x g. The precipitated protein formed a tight white pellet. The supernatant containing the DNA was then carefully removed (leaving the protein pellet behind) and transferred into a clean 1.5ml microcentrifuge tube containing an equal volume of isopropanol at RTM. The solutions were then mixed gently by inversion until the white thread-like strands of DNA formed a visible mass. The solutions were centrifuged for 1 min at 14,000 x g at RTM. The supernatant

was carefully decanted and the DNA was visible as a small white pellet. Ethanol (70%) at RTM was then added and the tubes gently inverted several times to wash the DNA. The solutions were centrifuged again for 1 min at 14,000 x g at RTM. Ethanol was then carefully aspirated using a Pasteur pipette and the pellet air dried for 10-15 min. The DNA was then rehydrated by adding DNA rehydration solution (Promega) and incubating at 65°C for 1 h. DNA was then analyzed by PCR to determine the genotype of the animal. Each sample was amplified in two different PCR reactions, one to determine the presence of the wild-type *Hoxa2* allele (5´-GTTGGAACTGACCTCCTCTTG-3´ and 5´-GGGTCCGAGCAGGGTTATTCC-3´) and the other to detect the presence of the neomycin cassette that replaces part of exon 1, all of intron 1 and part of exon 2 in the knockout animals (5´-TCGCCTTCTATCGCCTTCTTG-3´ and 5´-GTTGGTGTACGCGGTTCTCAG-3´). PCR reactions (50 µl) containing 10X amplification buffer (Invitrogen), 2 mM dNTP mix (Sigma), 1µM of each primer (Invitrogen) and 2.5 U of Taq polymerase enzyme (VWR) and 1 µg of DNA were prepared. Reactions were run on the BioRad MyCyclerTM using the temperature cycling conditions 95°C for 4 min followed by 35 cycles of 95°C for 30 s, 65°C for 3 min and 72°C for 7 min (Gendron-Maguire et al., 1993). PCR samples were then separated on a 1% agarose gel containing Redsafe™ (Frogga Bio) and visualized using UV light.

5.3 Immunohistochemistry (IHC)

Embryos were harvested from timed-pregnant mice, and heads were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH7.4) for 24 h. Embryo heads were then placed in 30% sucrose in PBS for at least 24 h (or after sinking to bottom of well) before being embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) and serially sectioned (in a coronal

plane) on to 0.5% gelatin coated glass slides. Determination of anterior, medial and posterior was performed by matching to sections from each region (Rice et al., 2004; Smith et al., 2013). Sections were then allowed to dry at RTM for at least 2 h. The 8 µm thick sections were then rehydrated for 45 min in 1xPBS and blocked in 4% skim milk powder and 0.1% Triton X-100 in PBS for 30 min at RTM. Sections were then exposed to the primary antibody diluted in PBS overnight at 4°C using the following antibodies; *Six2* rabbit polyclonal antibody (Proteintech™; 1:500), E-cadherin rat monoclonal antibody (Sigma™ ; 1:200) and *Ki-67* rat monoclonal antibody (Affymetrix eBioscience; 1:200). Excess unbound primary antibody was rinsed off by two 5 min washes in PBS before being exposed to a labelled secondary antibody for 1.5 h at RTM (IgG Alexa fluor 488 antibody,1:200 or IgG Alexa fluor 594, 1:400; Invitrogen™). Finally, sections were rinsed two more times in PBS, mounted in ProLong® Gold antifade reagent with DAPI (Molecular Probes) and viewed by fluorescence microscopy. To ensure that the signal observed was not the result of non-specific signal from the secondary antibody, negative controls were performed. These included staining in the absence of the primary antibody, and another set where the secondary antibody was omitted.

5.4 RNA Isolation and Reverse Transcription

Total RNA was isolated from excised wild-type and *Hoxa*2 null embryo palate shelves using the RNeasy® Protect Mini Kit (Qiagen) as per the manufacturer's protocol. RNA concentration was determined by optical density (Nanovue, General Electric). First strand DNA synthesis was performed using the SuperScript® first-strand synthesis system for RT-PCR (Invitrogen), using random decamer primers, as per the manufacturer's protocol. Final concentration of RNA for all RT reactions was 20 ng/µl.

5.5 Quantitative Real Time RT-PCR

Gene expression analysis was performed as previously described by Smith et al., 2009. Briefly, Taqman® primers and labelled probe systems (Applied Biosystems) and a real time PCR machine (ABI 7300) from Applied Biosystems were used for real time PCR analysis of *Six2*, *Msx1*, *Bmp4*, *Barx1* and *Ptx1*. Palatal shelves were aseptically dissected from both wild-type and *Hoxa*2 null mice at stages E12.5, E13.5, E14.5 and E15.5. All reactions were performed using the Taqman Universal Master Mix (2X), FAM-labelled Taqman Gene Expression assays for *Six*2, *Msx1, Bmp4, Barx1 and Ptx1*, VIC-labelled Taqman Endogenous Control β-Actin, and 10ng of cDNA. All reactions were run in replicates of 5 (5 samples, each from a different embryo). Thermocycling parameters were as follows: 2 min at 50^{o}C, 10 min at 95 ^{o}C, 40 cycles of 15s at 95 ^{o}C followed at end with one cycle at 60 ^{o}C for 70s. Taqman gene expression assays have all been tested to have efficiencies not significantly different from 1 (Applied Biosystems, 2004). However, in order to ensure that all primer sets were working properly and that multiplexing the gene specific and β-Actin primers did not result in altered amplification efficiencies all primer sets were examined individually and in complex with β-Actin.

5.6 Droplet digital PCR (ddPCR)

The procedure for PCR analysis using droplet digital PCR was performed as described by Hindson et al., 2013. Briefly, each 20 µl PCR reaction was loaded into an 8-channel, single-use consumable droplet generation cartridge. Oil (60 µl) containing emulsion-stabilizing, biocompatible surfactant was loaded into adjacent oil wells and the microfluidic chip was loaded into a beta-series prototype droplet generator (DG). The DG applies a vacuum to the

outlet well creating a pressure difference across the cartridge that simultaneously partitions the sample present in each of the 8 wells into approximately 20,000 monodisperse droplets of accurately known volume. This process occurs at a rate of approximately 1,000 droplets/s per well. The resulting water-in-oil emulsions were pipette-transferred from the outlet well to a 96-well polypropylene plate (Eppendorf), sealed with foil and then amplified to endpoint using a Tetrad2 Peltier Thermal Cycler (Bio-Rad) and the cycling protocol: 95 °C for 10 min then 40 cycles of 95 °C for 15 s and 60 °C for 1 min (2.5 °C/s ramp rate) with a final 10 min hold at 98 °C. Plates containing amplified droplets were loaded into a QX 100 droplet reader (Bio-Rad), which aspirates droplets from the 96-well plate, one well at a time, and streams them single file (about 1,500 droplets/s) past a two-color detector of FAM and VIC fluorescent dyes (Life Technologies) sampling at 100 kHz. Discrimination between droplets that did not contain target (negatives) and those that did (positives) was achieved by applying a global fluorescence amplitude threshold. Concentration estimates were based on the fraction of droplets where amplification has occurred modelled as a Poisson distribution.

5.7 Western blot analysis

Palatal shelves were aseptically dissected from both wild-type and *Hoxa*2 null mice before being pooled together prior to lysis in radio-immuno precipitation assay [RIPA] buffer containing protease inhibitor (150 mM NaCl, 10 mM Tris, 0.1%-SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA). Protein was quantified using the Bradford assay to ensure that equal loading was achieved. Samples were then boiled for 15 min with loading buffer and loaded on a 10% polyacrylamide-SDS gel. After gel separation, the proteins

were transferred to a PolyScreen PVDF membrane, which was subsequently blocked by overnight incubation in 5% skim milk in PBS (SM-PBS) at 4°C.

The membrane was then exposed to a *Six2* primary antibody (*Six2* rabbit polyclonal, Protein tech, 1:2000 in 5% skim milk in PBS) and incubated for 1 h at RTM. This was followed by 4 consecutive washes of 15 min each in PBS with 0.08% Tween-20. After the washes were complete, the membrane was incubated with a secondary antibody specific to the species of the primary antibody that is used (anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) 1:3000) in SM-PBS for 1 h at RTM. After 4 washes of 15 min each in PBS-Tween-20, membrane was exposed with a chemiluminescence reagent (ECL substrate, BIORAD) and the signal was detected using the SYNGENE imager. The membrane was then washed overnight at 4°C in PBS before being incubated with the anti-β-tubulin (Development Studies Hybridoma Bank, U. Iowa) at a dilution of 1:2000, and incubated for 1 h at RTM, followed by 4 washes of 15 min each in PBS with 0.08% Tween-20. After the washes, the membrane was exposed to the anti-mouse IgM horseradish peroxidase conjugate (Bio-Rad) in SM-PBS at a dilution of 1:1500, and then treated as indicated above.

After imaging, semi-quantitative densitometry was performed using the AlphaImager® densiometric software to determine an integrated density value (IDV) for each band. Four separate western blot assays, each having wild-type and *Hoxa2* null samples of E12.5, E13.5, E14.5, E15.5 were performed. The software allowed for the selection of a representative background area and then to define an area around the bands of interest. Equal size boxes were used in all comparisons. To compare expression, relative IDV values for each blot were calculated by normalizing to β-tubulin levels from the same sample. The resulting values (wild-type and *Hoxa2* null of E12.5, E13.5, E14.5, E15.5)

were then normalized to the E12.5 wild-type sample of that blot. Due to differences in the IDV values between different blots it was necessary to normalize each blot to a band on its own gel to accurately represent any changes in protein expression observed. A total of four blots (n = 4) were analyzed using two way ANOVA to compare Six2 protein expression in wild-type and *Hoxa2* null samples at (E12.5, E13.5, E14.5 and E15.5).

5.8 Culturing MEPM cells

Mouse embryonic palate mesenchyme (MEPM) cells were cultured as previously described by Iwata et al., 2012. Briefly, timed pregnant C57 black mice were sacrificed at stage E13.5 and their embryos removed. Palatal shelves were aseptically excised from the embryo and placed in Hank's balanced salt solution. A piece of tissue was also taken from the embryo for PCR genotyping analysis. In addition, the palatal shelves were treated according to Tyler et al., 1980, to separate the palatal mesenchyme from the epithelium. Purified palatal mesenchyme was incubated with 0.25% trypsin/0.02% EDTA in Ca^{++}-Mg^{++} free PBS for 20 min at 37°C to dissociate individual cells. The digested samples were then briefly triturated and filtered through a 70 μm mesh. The action of trypsin was terminated using 4°C Dulbecco's Modified Eagle's Medium (DMEM)/Ham's medium F12 (1:1) containing 10% fetal bovine serum (FBS) supplemented with 1% penicillin/streptomycin, L-glutamate, sodium pyruvate and non-essential amino acids. The cell number was determined using the MOXI Z automated cell counter (Orflo Technologies) and the primary culture of MEPM cells was initiated by seeding $5x10^5$ cells/5ml of complete DMEM/F-12 media in a 25ml tissue culture flask. Cells were sub cultured at a ratio of 1:3 after achieving confluence in a 95% air and 5% CO_2 atmosphere.

5.9 siRNA treatment and cell proliferation analysis

The siRNA used was the pre-designed *Six2* Silencer Select siRNA (Invitrogen: catalogue number 4390771), together with a Silencer select negative control siRNA (Invitrogen). siRNA aliquots were prepared by diluting the stock siRNA (5nM lyophilized powder) using nuclease-free sterile water (Invitrogen) to achieve a working concentration of 20μM. MEPM cells ($5.0x10^3$) were plated in a 96-well plate until they reached 60%-80% confluence. The siRNA sample was mixed with lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Briefly, for each transfection, 0.3μl of lipofectamine 3000 (Invitrogen) was diluted with 5μl of serum free DMEM/F12 media and kept at RTM for 5 min. This was mixed with 5μl of serum free media containing 0.25μl of siRNA and the mixture vortexed for 10 sec and left at RTM for 20 min. Prior to transfection, cells were rinsed twice with serum free media. The above siRNA-lipofectamine complex (10μl) was added to each well of MEPM cells containing 100μl of serum free media. Following incubation at 37°C for 12 h, the transfection media was replaced with fresh complete media.

MEPM cells were analyzed for cellular DNA content 48 h from the time of transfection using the CyQUANT NF cell proliferation assay kit (Life Technologies). The cell proliferation assay was performed as per the manufacturer's protocol. Briefly, the transfected MEPM cells were incubated with 1X dye binding solution at 37°C for 45 min in the dark. Fluorescence was detected with a microplate reader (BioTek) with excitation at 485nm and emission at 530nm.

5.10 Statistical analysis

All statistical analyses and graphs were performed using the GraphPad 5.0 software package (GraphPad Prism). Statistical analysis on quantitative real time RT-PCR data for *Six2* expression was evaluated using two-way analysis of variance (2 way ANOVA) followed by Bonferroni post-hoc tests to compare between the genotypes and embryonic stages.

6. RESULTS

6.1 Six2 protein exhibits a temporal distribution pattern in the developing secondary palate

In order to investigate if *Six2* might be involved in palatogenesis, I first examined whether this gene was endogenously expressed during the different stages of palatogenesis: emergence and vertical outgrowth of palatal shelves (E12.5-E13.5); palatal shelf elevation and horizontal growth (E14.0-E14.5); and palatal shelf fusion (E15.0-E15.5). I utilized fluorescence immunohistochemistry (IHC) to demonstrate that Six2 protein is expressed differentially across the four stages of secondary palate development (E12.5, E13.5, E14.5, E15.5). Immunohistochemical analysis of coronal sections from the anterior, medial and posterior regions of wild-type embryos showed that Six2 protein was initially high at E12.5, increased to peak expression at E13.5 and reduced at E14.5 before further declining at E15.5 (Fig. 9).

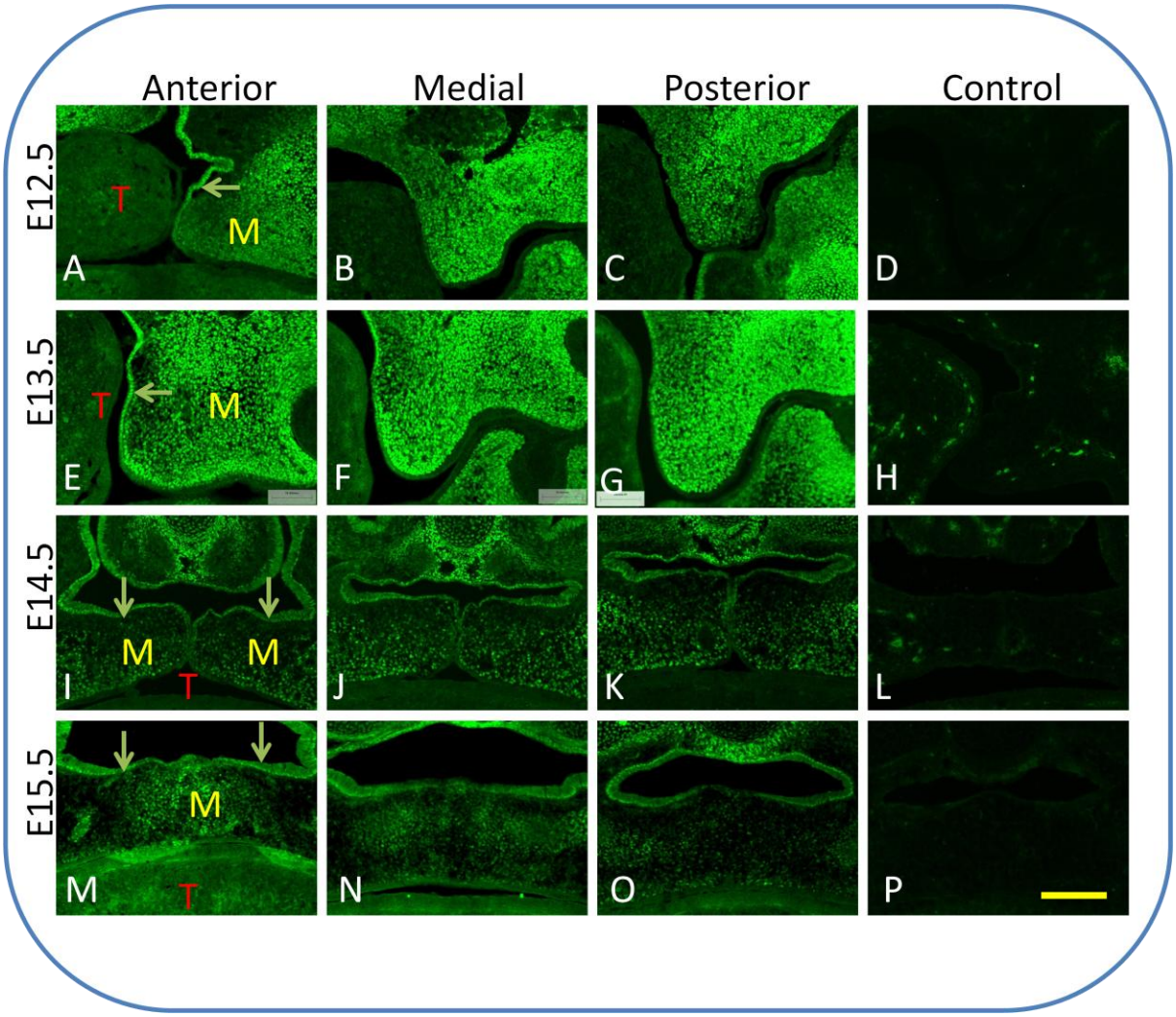


Figure 9: Six2 protein exhibits a temporal distribution pattern in the developing palate. Immunohistochemical staining of coronal sections from the anterior, medial and posterior regions of wild type embryos showing more staining of Six2 protein at earlier stages of palatogenesis (E12.5-E13.5) and less staining at the later stages (E14.5-E15.5). Panels D, H, I, P are control sections where only the secondary antibody was used. Anterior, medial, posterior and control sections are all from the same embryo. Arrow indicates epithelium, T-tongue, M-mesenchyme, 10X magnification. (scale bar, 10 µm).

6.2 Six2 protein is spatially distributed in both the epithelium and mesenchyme of the developing secondary palate

Immunohistochemical analyses of the anterior, medial and posterior sections from both wild-type and *Hoxa2* knock out embryos revealed that Six2 protein was consistently expressed in both the epithelium and mesenchyme of mouse palatal shelves across the stages of palatogenesis. It was interesting to note that Six2 protein was expressed in the nasal palatal epithelium (Fig. 10B) but was completely absent from the oral palatal epithelium (Fig. 10D). This expression pattern was consistent in the A-P axis of the palate and across the stages of secondary palate development. To confirm this finding, I double-labelled palatal sections by IHC using *Six2* and *E-cadherin* antibodies. *E-cadherin* is a known epithelial marker that stains only in the epithelium. Cells in the nasal palatal epithelium were positive for both *Six2* and *E-cadherin* staining (Fig. 10J), while cells in the oral palatal epithelium were only positive for *E-cadherin* (Fig. 10L). This confirmed my earlier observation that Six2 protein was only expressed in the nasal palatal epithelium and was completely absent from the oral palatal epithelium.

Furthermore, I observed more Six2 protein expression in the oral half of the palatal mesenchyme as compared to the nasal half of the palate mesenchyme (Fig. 11). This pattern of expression was again consistent in the A-P axis of the palate and from E12.5 to E14.5. At E15.5 however, Six2 protein expression seemed to be concentrated in the central portions of the palatal mesenchyme when compared to the oral and nasal halves of the palatal mesenchyme where expression was less.

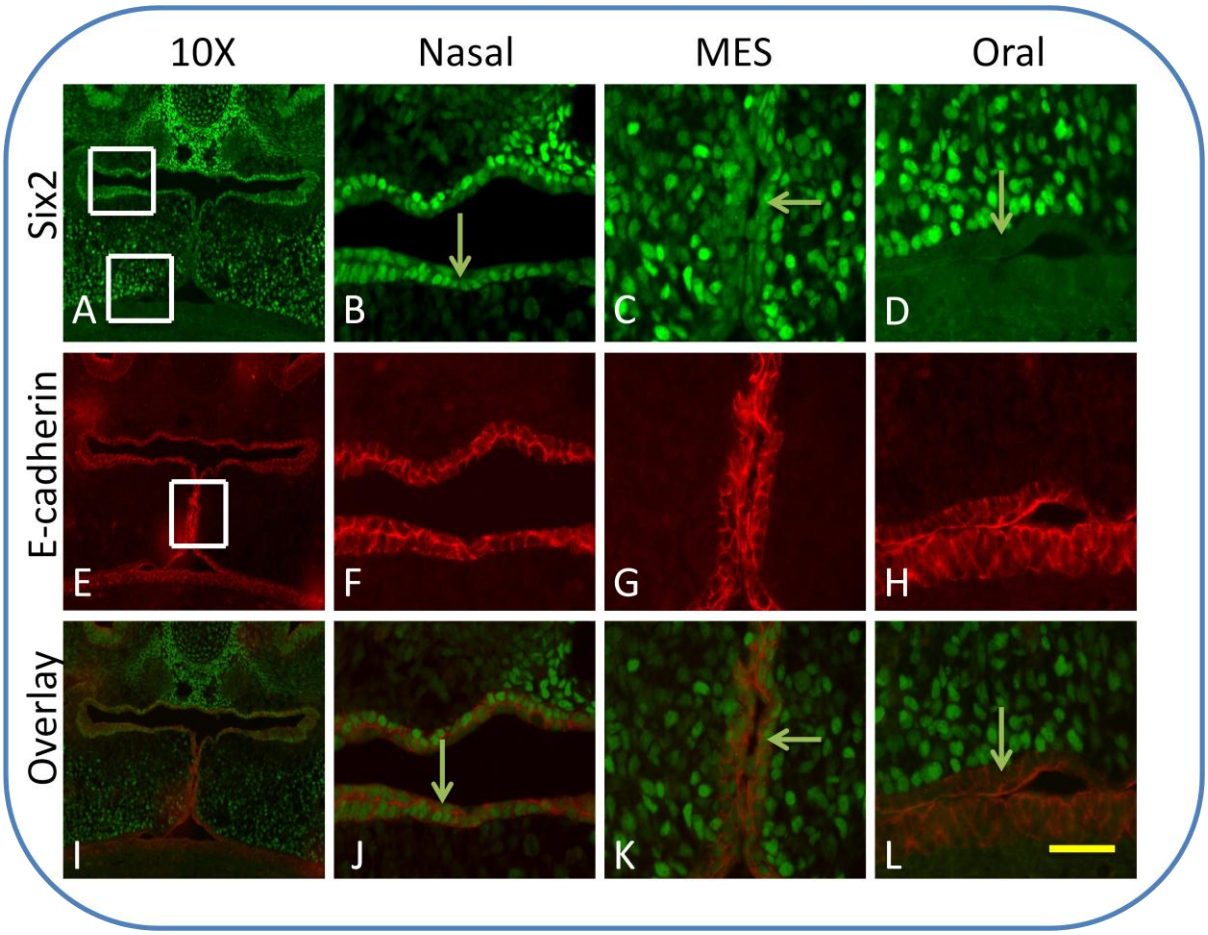


Figure 10: Six2 protein exhibits a spatial expression pattern in the palate epithelium. Immunohistochemical double-labelling of Six2 protein (A,B,C,D) and of E-cadherin (E,F,G,H) on coronal sections of the developing palate at E14.5. Staining of Six2 protein can be observed in the nasal epithelium (B), reduced staining in the region of the midline epithelial seam (MES) (C) and no staining in the oral epithelium (D). Blue boxes in panels A, E represent magnified regions (40X) for the MES, nasal and oral epithelium as shown in panels B,C,D,F,G,H,J,K & L . Sections I,J,K,L are overlays. (Scale bar, 10µm).

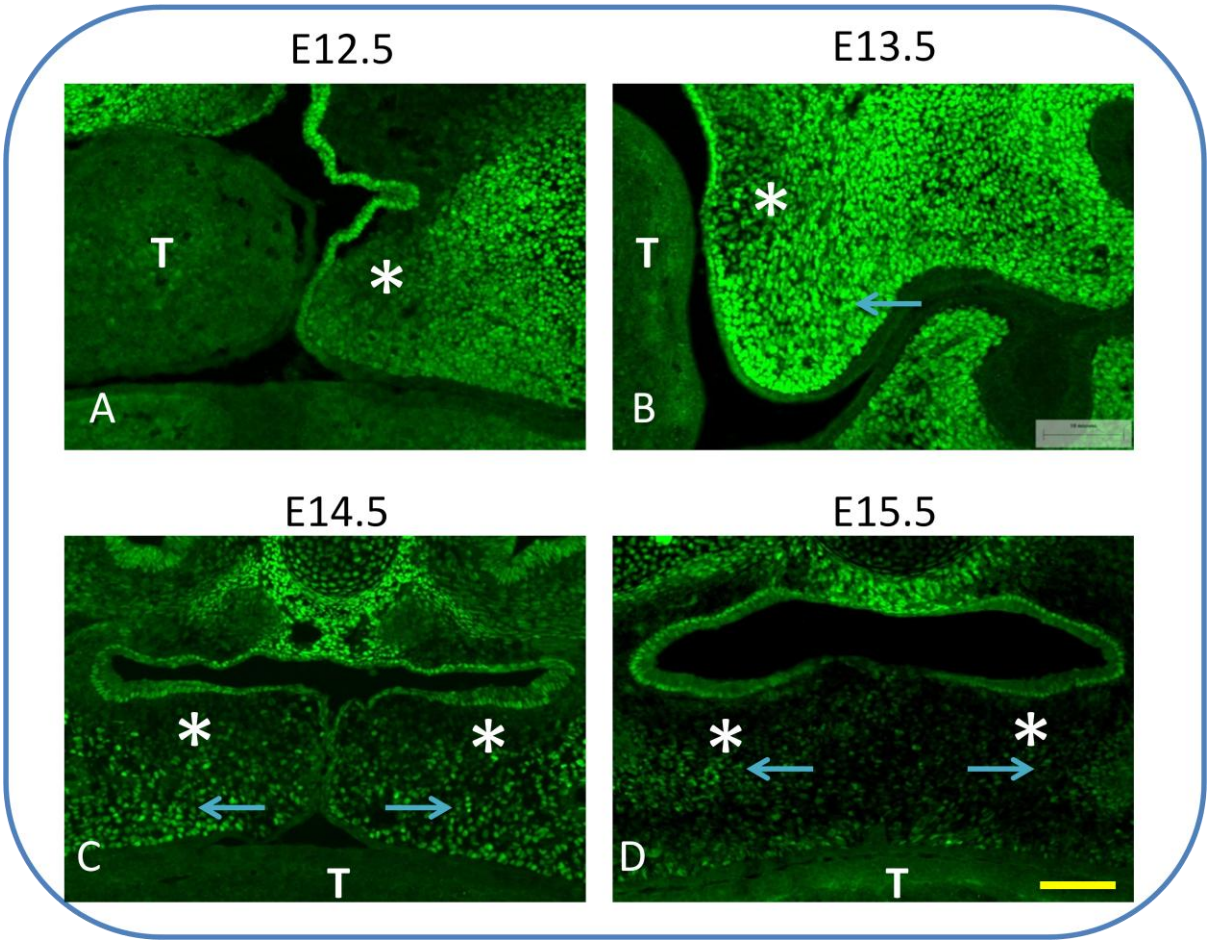


Figure 11: Six2 protein exhibits a spatial expression pattern in the palatal mesenchyme. Immunohistochemical staining of representative coronal sections of the developing secondary palate of wild-type embryos (E12.5-E15.5). From E12.5 to E14.5, there is more staining for Six2 protein in the oral half of the palatal mesenchyme (arrows) and less staining in the nasal half of the palatal mesenchyme (asterisks) (A-C). At E15.5, Six2 protein expression is concentrated in the central portions of the palatal mesenchyme (arrows) (D). T-tongue.(10x Magnification) (Scale bar, 10µm).

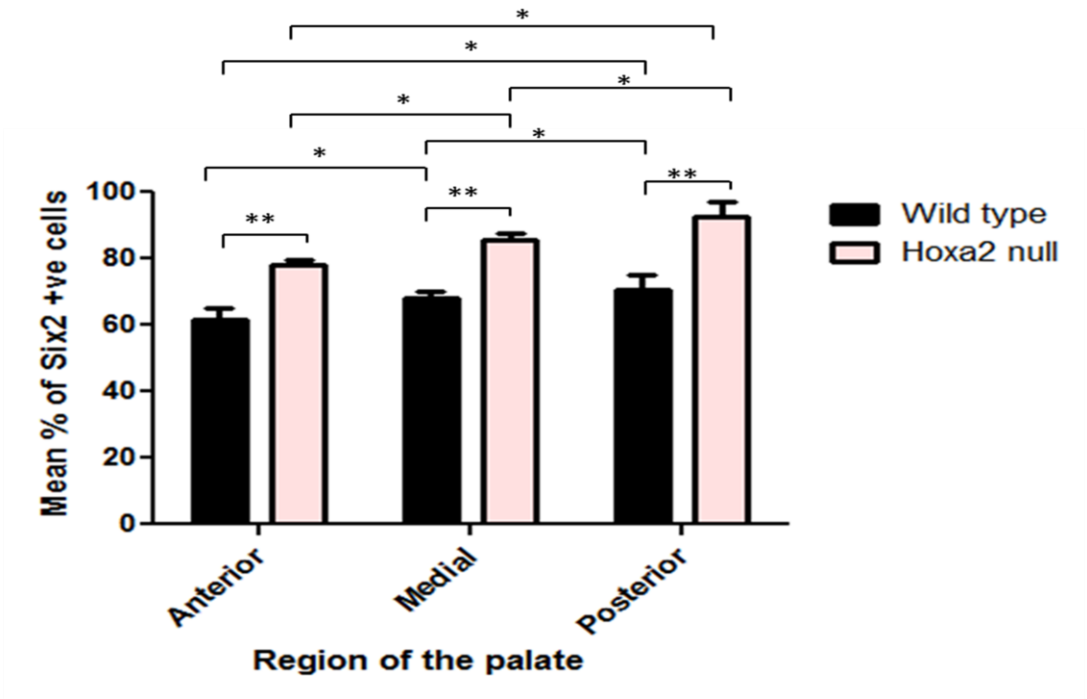
6.3 Six2 protein exhibits an A-P differential expression pattern in the developing secondary palate

Analysis of the anterior, medial and posterior regions of the palatal epithelium and mesenchyme revealed a differential expression pattern of Six2 protein in the A-P axis in both the wild-type and *Hoxa2* null embryos. *In vivo* cell counting analysis revealed a general increase in the A-P direction of the number of palate mesenchymal cells expressing Six2 protein, with the least expression in the anterior and most expression in the posterior regions of the palate (Fig. 12A).

In addition, IHC analysis showed increasing Six2 protein expression in the A-P direction with the most expression observed in the posterior portions of the palate mesenchyme (Fig. 12B). This expression pattern was consistent for both wild-type and *Hoxa2* null embryos and across the stages of secondary palate development. Also interestingly, it seems that Six2 protein is primarily expressed in the anterior nasal palatal epithelium and it progressively decreases as one moves into the posterior regions where little or no expression is seen in the posterior nasal palatal epithelia (Fig. 12B).

A)

In vivo cell counting analysis of Six2 positive cells at E13.5



B)

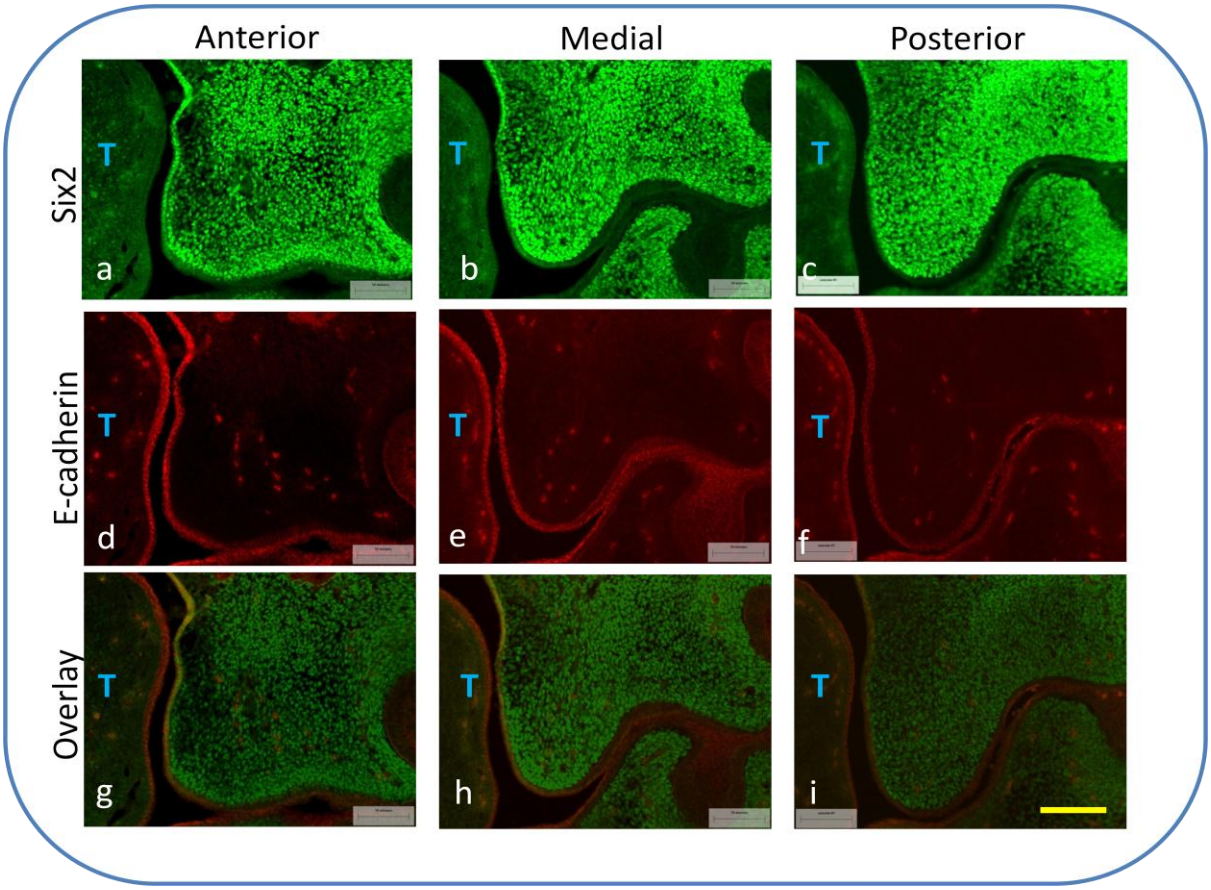


Figure 12: Six2 protein exhibits an Anterior-Posterior differential expression pattern in the palate epithelium and mesenchyme.

A) *In vivo* cell counting analysis of Six2 protein positive cells from coronal anterior, medial and posterior sections of wild-type and *Hoxa2* null mice (E13.5) showing highest Six2 protein expression in the posterior region of the palatal mesenchyme of *Hoxa2* null embryos.

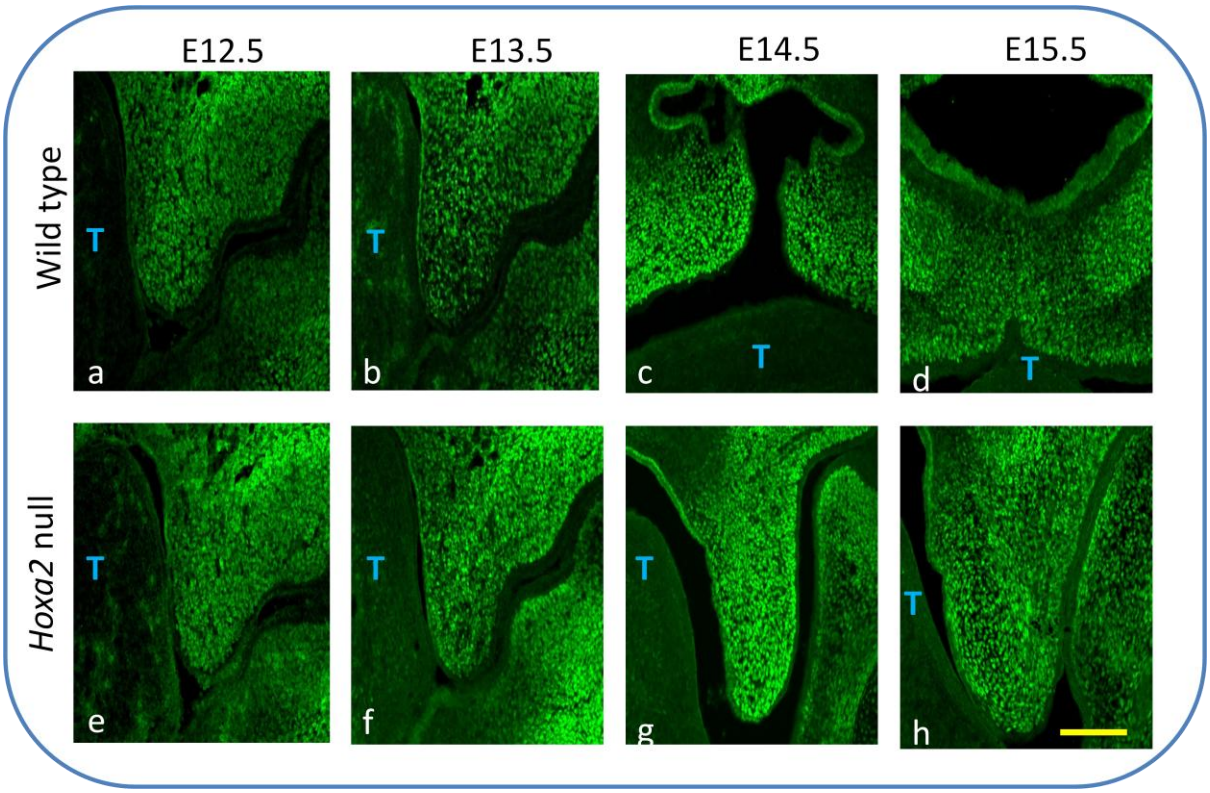
B) Representative coronal sections from the anterior, medial and posterior regions of wild type embryos (E13.5) showing Six2 protein expression the palatal mesenchyme increasing from the anterior towards the posterior region of the secondary palate. It is interesting to observe that in the posterior regions, expression of Six2 in the nasal palatal epithelium is also much lower compared to the anterior and medial epithelia. This pattern of expression is observed across all stages of palatogenesis and in both wild-type and *Hoxa2* null mice. Six2 positive cells were expressed as a percentage of the total cell number (DAPI positive cells). Bars represent mean ± SEM, n=4, *$p<0.05$, **$p<0.01$. T-tongue. (scale bar,10 μm).

6.4 *Six2* protein and mRNA are up-regulated in the absence of *Hoxa2*

The *Hoxa2* gene has been reported to bind to the *Six2* promoter *in vivo* to repress its expression (Kujetova et al., 2008). To confirm these findings, I performed quantitative real time PCR (qPCR) and western blot analysis on palatal shelves from both wild-type and *Hoxa2* null embryos. There was a significant increase in the expression of *Six2* at both the mRNA and protein level in the *Hoxa2* null embryos when compared to their wild-type littermates at all stages of palatogenesis, with peak expression occurring at E13.5 (Figs. 13B, C). The peak expression of *Six2* at E13.5 coincides with the time of palatal shelf vertical outgrowth on either sides of the tongue. In addition, I also performed absolute mRNA quantification by droplet digital PCR (ddPCR) to compare between ddPCR and qPCR (Appendix Fig.1). The results for mRNA quantification using ddPCR and qPCR matched indicating consistency of results using the two different techniques (Fig.13C and Appendix Fig.1).

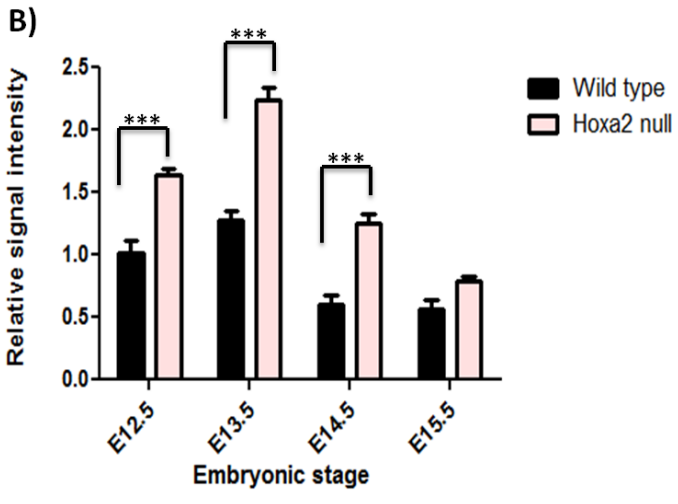
Furthermore, IHC analyses of anterior, medial and posterior sections of both wild-type and *Hoxa2* null embryos across the stages of palatogenesis revealed more Six2 protein expression in the *Hoxa2* null embryos compared to their wild type littermates (Fig. 13A). Additionally, *in vivo* cell counting analysis of Six2 protein positive cells in the nasal half of the palatal mesenchyme in wild-type and *Hoxa2* null littermates revealed an expanded domain of Six2 protein expression in the *Hoxa2* null palatal shelves. In the wild-type palatal shelves, Six2 protein expression is seen mainly in the oral half of the palatal mesenchyme with less expression observed in the nasal half of the palatal mesenchyme. In the *Hoxa2* null palatal shelves however, the domain of Six2 protein expression expands from the oral half of the palatal mesenchyme to include the nasal half of the palatal mesenchyme (Fig. 14).

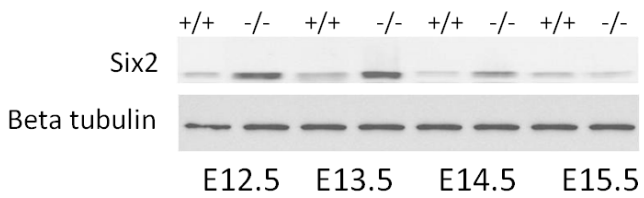
A)



Six2 western blot analysis

B)





Six2 mRNA qPCR analysis

C)

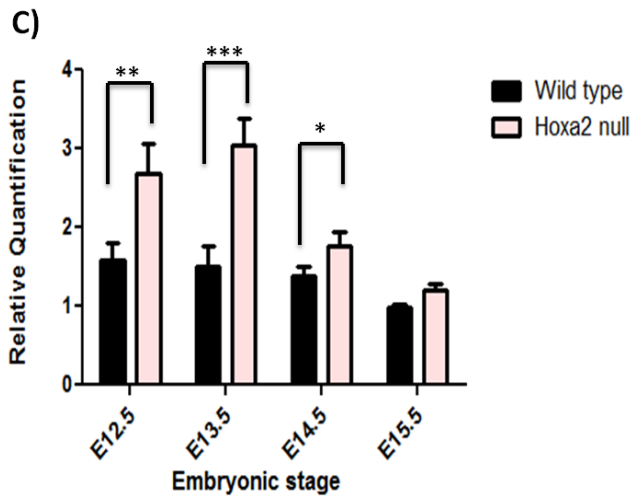


Figure 13: *Six2* mRNA and protein are up-regulated in the absence *Hoxa2.*

A) The domain of Six2 protein expression is expanded in the *Hoxa2* null palatal shelves. Immunohistochemical staining of representative medial coronal sections of the secondary palate from wild-type and *Hoxa2* null littermates showing an extended domain of mesenchymal Six2 protein expression in the *Hoxa2* null palatal shelves (E, F, G, H) compared to wild type palatal shelves (A, B, C, D). In the *Hoxa2* null palatal shelves, the domain of Six2 expression expands from the oral half of the palatal mesenchyme to include the nasal half of the palatal mesenchyme. Also, the palatal shelves fail to elevate above the tongue at E14.5 and E15.5 in the *Hoxa2* null embryos. T-tongue. (scale bar, 10μm).

B) Western blot analysis of protein obtained from palatal shelves of wild-type and *Hoxa2* null mice. There is enhanced Six2 protein expression in the absence of *Hoxa2* across all stages of palatogenesis with peak expression occurring at E13.5 in both wild-type and *Hoxa2* null palatal shelves.

C) Relative quantitative expression of *Six2 mRNA* during murine palatogenesis in wild-type and *Hoxa2* null mice. In the wild type mice, *Six2* mRNA expression is comparably high at E12.5 and E13.5 and reduces at E14.5 before decreasing further at E15.5. In the *Hoxa2* null mice, expression is enhanced across all stages of palatogenesis. In the absence of *Hoxa2, Six2* mRNA is initially high at E12.5 and increases to a peak level at E13.5. This expression dramatically reduces at E14.5 before decreasing further at E15.5. Bars represent mean ± SEM, n=5, *$p<0.05$, **$p<0.01$.

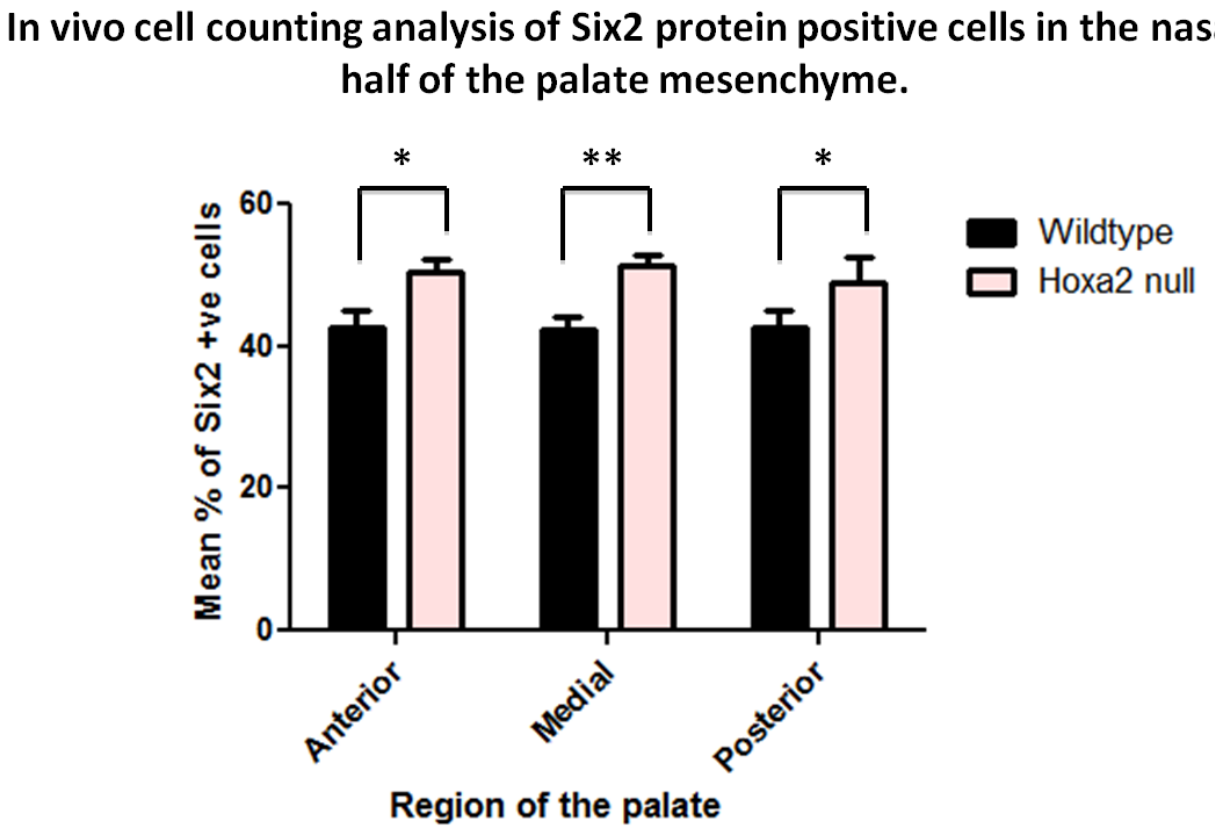


Figure 14: *In vivo* cell counting analysis of Six2 protein positive cells in the nasal half of the palatal mesenchyme in wild-type and *Hoxa2* null littermates (E14.5) showing an expanded domain of Six2 protein expression in the *Hoxa2* null palatal shelves. In the wild-type palatal shelves, Six2 protein expression is seen mainly in the oral half of the palatal mesenchyme with less expression observed in the nasal half of the palatal mesenchyme. In the *Hoxa2* null palatal shelves however, the domain of Six2 protein expression expands from the oral half of the palatal mesenchyme to include the nasal half of the palatal mesenchyme. Six2 positive cells were expressed as a percentage of the total cell number (DAPI positive cells). Bars represent mean ± SEM, n=4, *$p<0.05$, **$p<0.01$.

6.5 Analysis of cell proliferation in vivo

To investigate whether *Six2* has a role to play during palatal mesenchyme cell proliferation, I performed IHC analyses on anterior, medial and posterior palatal sections of both wild-type and *Hoxa2* null embryos, using an antibody for the Ki-67 antigen, a known marker of cell proliferation. This involved counting cells in a whole palatal shelf including; the total number of cells (DAPI positive cells), cells expressing the Six2 protein (Six2 positive cells), actively proliferating cells (Ki-67 positive cells) and the cells that were actively proliferating and co-expressing the Six2 protein (cells positive for both Six2 and ki-67). I chose to investigate this putative role of *Six2* at embryonic stage (E) 13.5 when the palatal shelves start growing vertically downwards on either side of the tongue. Incidentally, this is also the stage where I observed peak expression of the *Six2* gene at both the mRNA and protein level (see Figs. 13A, B, C).

I observed a significant increase in cell proliferation in the *Hoxa2* null palatal shelves as compared to the wild-type palatal shelves in both the A-P axis and the O-N axis of the palatal mesenchyme, with the highest percentage of proliferating cells observed in the nasal half of the posterior palatal mesenchyme (Figs. 15A-G). In addition, the percentage of proliferating cells that were also expressing the Six2 protein (Six2/ki-67 double positive cells) was significantly increased in the *Hoxa2* null palatal shelves when compared to the palatal shelves from wild-type embryos, in both the A-P axis and the O-N axis of the palate mesenchyme. Again, the highest percentage of these double positive cells was observed in the nasal half of the posterior palatal mesenchyme (Figs. 16A-G).

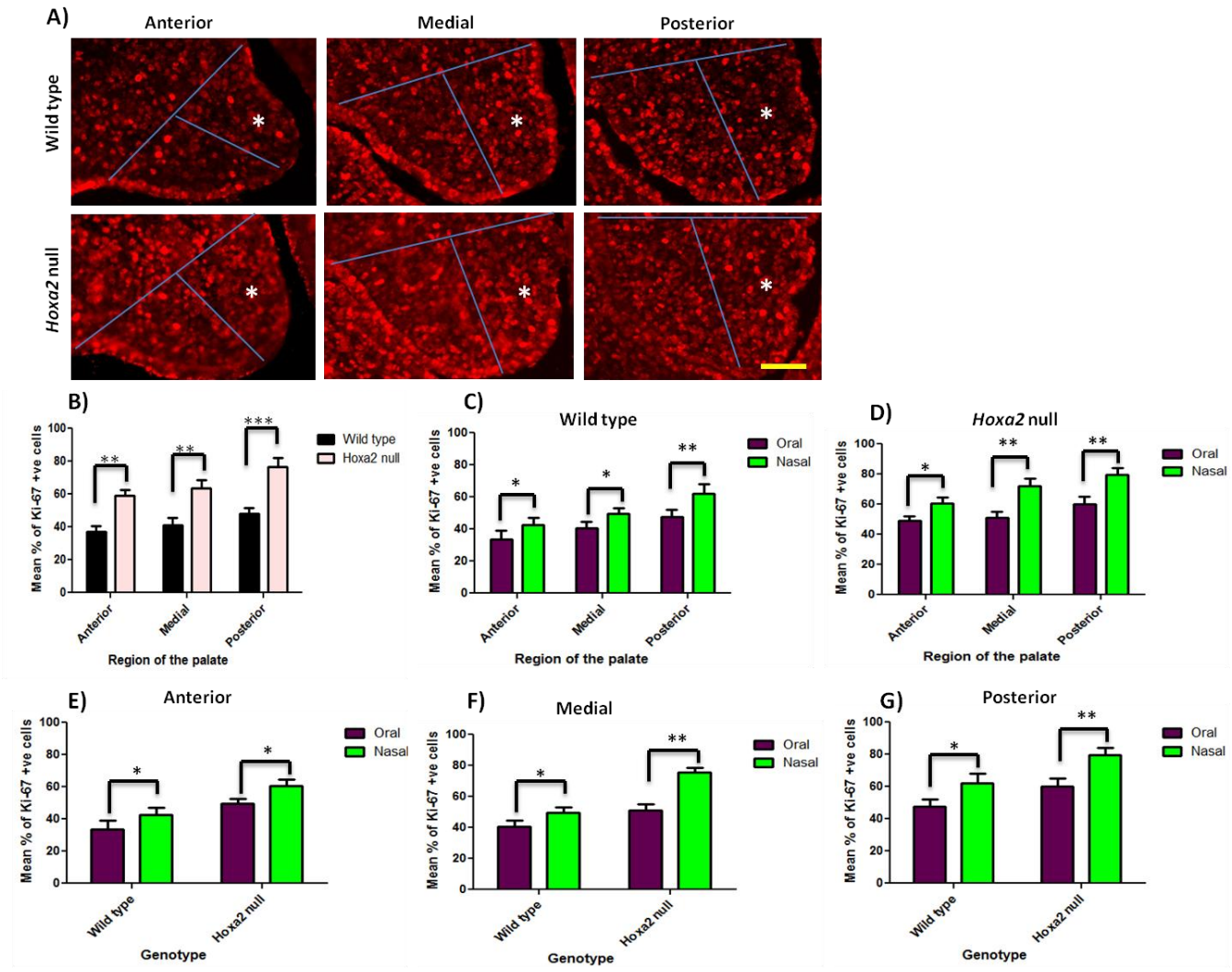


Figure 15: Increased cell proliferation in nasal half of the posterior palatal mesenchyme of *Hoxa2* null palatal shelves.

A) Immunohistochemical staining of coronal sections from the anterior, medial and posterior regions of wild-type and *Hoxa2* null palatal shelves (E13.5) for Ki-67, a marker of cell proliferation**. (A-G)** In the *Hoxa2* null palatal shelves, there is a significant increase in cell proliferation in the both the A-P axis and O-N axis, with the highest percentage of proliferating cells observed in the nasal half of the posterior palatal mesenchyme. Asterisks indicate the nasal half of the palatal mesenchyme. Proliferating cells were expressed as a percentage of the total cell number (DAPI positive cells). (scale bar,10 µm). Bars represent mean ± SEM, n=4, *p<0.05, **p<0.01.

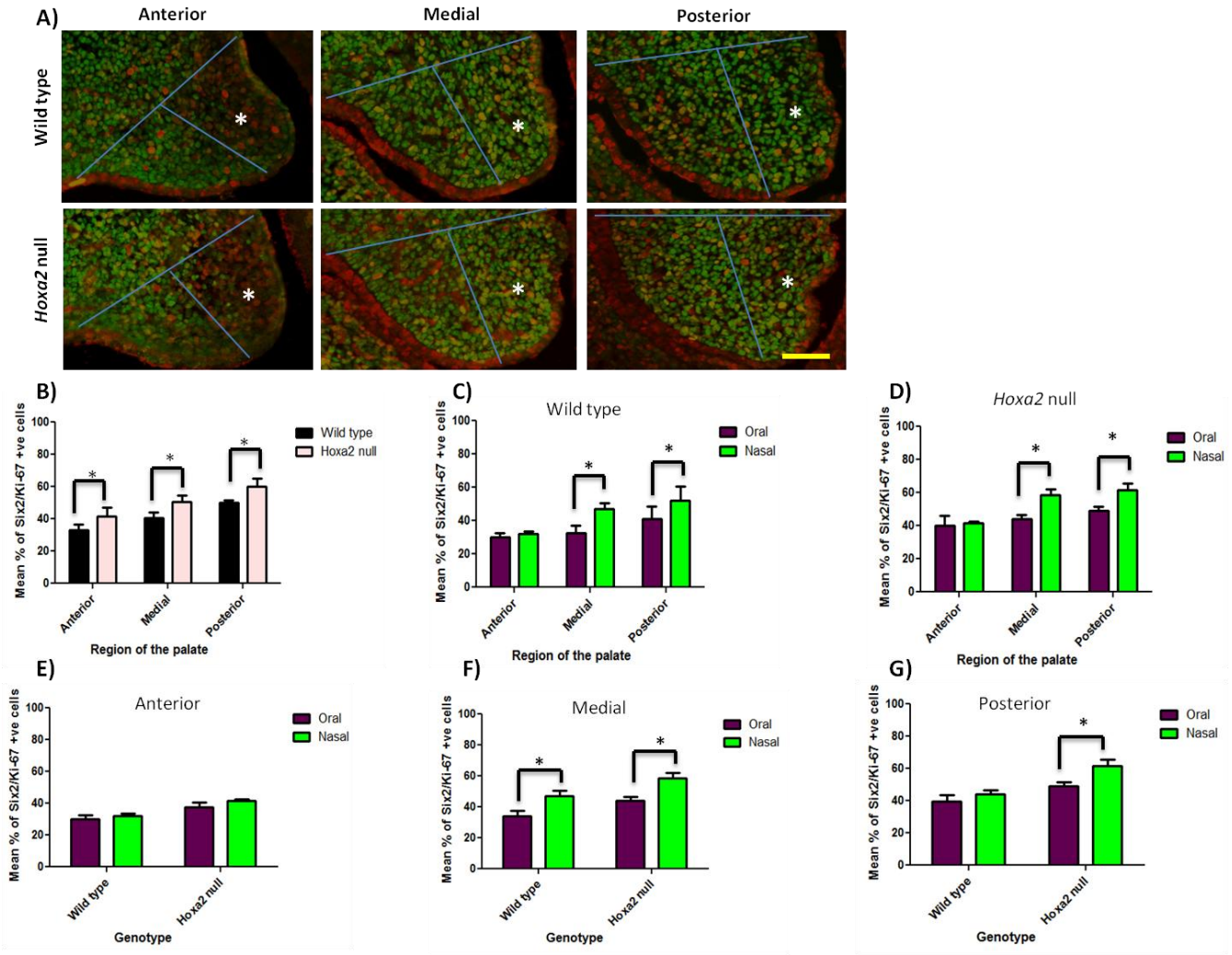


Figure 16: The number of cells co-expressing *Six2* and *Ki-67* is highest in the nasal half of the posterior palatal mesenchyme of *Hoxa2* null palatal shelves. **A)** Immunohistochemical staining of coronal sections from the anterior, medial and posterior regions of wild-type and *Hoxa2* null palatal shelves (E13.5) for both Six2 and Ki-67 (double staining). **(A-G)** There is a significant increase in the percentage of proliferating cells that are co-expressing Six2 protein (Six2/Ki-67 double positive cells) in the *Hoxa2* null palatal shelves in both the A-P and O-N axes of the palatal mesenchyme. The highest percentage of these double positive cells was observed in the in the nasal half of the posterior palatal mesenchyme. Asterisks indicate the nasal half of the palatal mesenchyme. Cells co-expressing Six2 and Ki-67 were expressed as a percentage of total Six2 positive cells (scale bar,10 µm). Bars represent mean ± SEM, n=4, *p<0.05, **p<0.01.

6.6 Analysis of cell proliferation in vitro

In addition to *in vivo* cell proliferation analysis, I also performed analyses of cell proliferation *in vitro*. This involved knocking down the expression of *Six2* mRNA in primary palatal cell cultures using *Six2* siRNA and observing the effect this had on cell proliferation and the expression of the cell cycle regulator, *Cyclin D1.* I used an MEPM cell culture system of both wild-type and *Hoxa2* null cells. These were freshly cultured primary cells prepared from the palate mesenchyme of E13.5 embryos, a stage at which palatal shelves grow vertically downwards on either sides of the tongue musculature.

In order to achieve *Six2 mRNA* knock down in MEPM cells, two *Six2* siRNA sequences were tested namely; sequence 1 and sequence 2. *Six2* siRNA sequence 1 achieved 90% knockdown of S*ix2* mRNA while *Six2* siRNA sequence 2 only achieved 30% knockdown of *Six2* mRNA (Fig. 17). I therefore chose to use *Six2* siRNA sequence 1 for my subsequent experiments.

In addition, I used the CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen) for in vitro analysis of cell proliferation. The CyQUANT® NF assay is based on measurement of cellular DNA content via fluorescent dye binding. Because cellular DNA content is highly regulated, it is closely proportional to cell number. The extent of proliferation was determined by comparing cell counts for siRNA treated MEPM cell cultures and the control cell cultures. The two controls used in my experiment were the mock control, where there was no siRNA treatment and the cell cultures treated with a scramble control siRNA from the same vendor (invitrogen) that is known not to cause *Six2* mRNA knockdown.

It was interesting to observe that *in vitro Six2* siRNA knock down restored cell proliferation in the *Hoxa2* null MEPM cell cultures to wild-type levels (Fig. 18A). In both the wild-type and *Hoxa2* null cell cultures, I observed

that cell proliferation was significantly reduced when they were treated with *Six2* siRNA compared to the mock and control siRNA cell cultures.

Furthermore, my study sought to find out if the knockdown of *Six2 in vitro* would have an effect on the expression of *Cyclin D1*. Indeed, I found that *in vitro Six2* siRNA knock down restored *Cyclin D1* expression in the *Hoxa2* null palatal mesenchyme cell cultures to wild-type levels (Fig. 18B). In both the wild-type and *Hoxa2* null palatal mesenchyme cell cultures, *Cyclin D1* expression was significantly reduced after *Six2* siRNA knock down compared to the mock and control siRNA treated cell cultures.

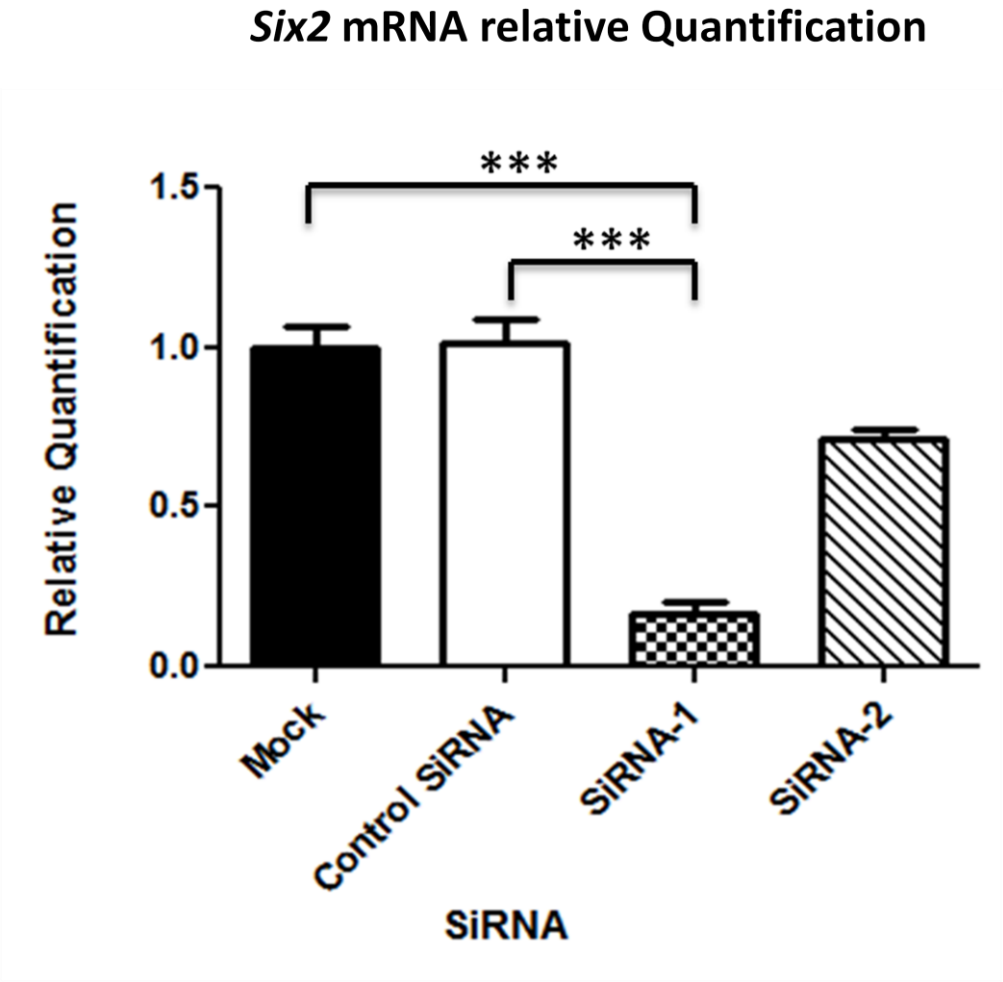


Figure 17: *Six2* siRNA knockdown in MEPM cells. 90% *Six2* mRNA knockdown was achieved using *Six2* siRNA sequence-1 compared to 30% *Six2* mRNA knockdown using *Six2* siRNA sequence-2. *Six2* siRNA sequence-1 was therefore chosen for the subsequent experiments. Mock treated cell cultures are those where there was no siRNA treatment, while the control siRNA cell cultures were treated with a scramble control siRNA. Bars represent mean ± SEM, n=4, *p<0.05, **p<0.01.

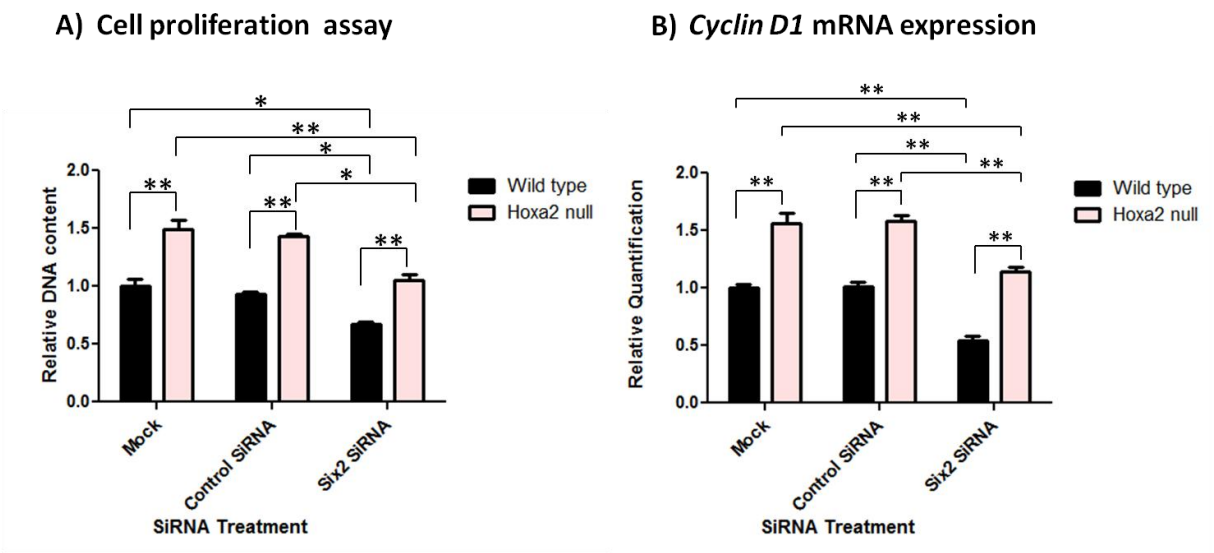


Figure 18: A) *In vitro Six2* siRNA knock down restored cell proliferation in the *Hoxa2* null palate mesenchymal cell cultures to wild-type levels. In both the wild-type and *Hoxa2* null MEPM cell cultures, proliferation was significantly reduced when they were treated with *Six2* siRNA compared to the mock and control siRNA cultures. **B) *In vitro Six2* siRNA knock down restored *Cyclin D1* expression in the *Hoxa2* null palate mesenchymal cell cultures to wild-type levels.** In both the wild-type and *Hoxa2* null MEPM cell cultures, *Cyclin D1* expression was significantly reduced when they were treated with *Six2* siRNA compared to the mock and control siRNA cultures. Mock treated cell cultures are those where there was no siRNA treatment, while the control siRNA cell cultures were treated with a scramble control siRNA. Bars represent mean ± SEM, n=4, *p<0.05, **p<0.01.

During early embryonic development, a number of genes and signalling molecules including*, Msx1, Bmp4, Fgf10, Barx1* and *Ptx1* are expressed in the developing secondary palate (Zhang et al., 2002, Yu et al; 2005, Alappat et al., 2005). In this study, I wanted to determine if *in vitro Six2* siRNA knock down in MEPM cell cultures would have an effect on the expression of *Bmp4, Msx1, Barx1* and *Ptx1*; some of the palatal mesenchyme cell signalling molecules expressed during palatogenesis. *In vitro Six2* siRNA knock down did not have an effect on the expression of the mRNA transcripts of these genes (Fig. 19). However, the expression of *Bmp4, Barx1* and *Ptx1* mRNAs was significantly enhanced in the *Hoxa2* null palatal mesenchyme cell cultures compared to the wild-type cell cultures. In addition, I observed that *Msx1* expression was significantly reduced in the *Hoxa2* null cell cultures compared to the wild-type cell cultures.

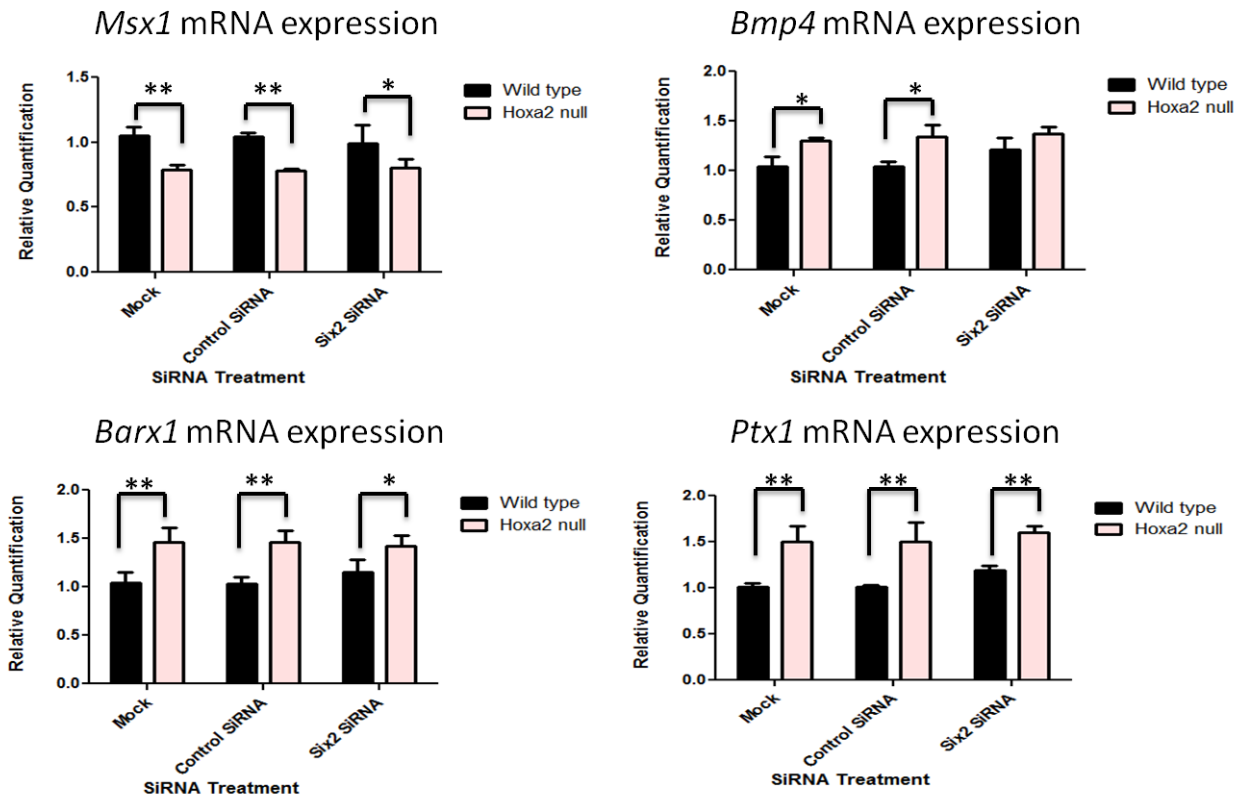


Figure 19: *In vitro Six2* siRNA knock-down did not have an effect on the expression of *Msx1, Bmp4, Barx1* and *Ptx1* mRNA transcripts. However, the expression of *Bmp4*, *Barx1* and *Ptx1* remained higher in the mock-treated control *Hoxa2* null MEPM cell cultures compared to the mock-treated control wild-type cell cultures. *Msx1* expression remained significantly reduced in the mock-treated control *Hoxa2* null cell cultures compared to the mock-treated control wild-type cell cultures. Bars represent mean ± SEM, n=4, *p<0.05, **p<0.01.

7. DISCUSSION

7.1 *Six2* exhibits a temporal-spatial expression profile in the developing secondary palate

In order to study the function of *Six2* in the developing secondary palate, my study first sought to establish if this gene is expressed in the mouse secondary palate. Indeed, *Six2* is expressed in the developing SP and my study has characterized for the first time the temporal and spatial distribution patterns of *Six2* in the developing secondary palate.

Using real time PCR and western blot analysis, I was able to demonstrate that *Six2* mRNA and protein respectively are expressed in the developing SP from the time of palatal shelf emergence at E12.5 to the time of palatal shelf fusion at E15.5 (Fig. 13B, C). In addition, fluorescence IHC on coronal sections from heads of wild-type mouse embryos revealed Six2 protein expression at all the stages of SP development (Fig.9). *Six2* expression at both the mRNA and protein level was higher during the earlier stages of palatogenesis (E12.5 and E13.5) compared to the later stages of palatogenesis (E14.5 and E15.5) (Fig. 9 and Fig. 13A-C). *Six2* expression is initially high at E12.5, it then increases to a peak expression at E13.5 before dramatically reducing at E14.5 and further declining by the time of palatal shelf fusion at E15.5. It is interesting to note that the peak expression of *Six2* at E13.5 coincides with the time when the paired palatal shelves start to grow vertically downwards on either sides of the tongue.

These findings have expanded on what earlier studies had reported that *Six2* is expressed in the craniofacial region of developing mouse embryos as early as E11.5 (Oliver et al., 1995; Brodbeck et al., 2004; Fogelgren et al., 2008). In addition, the high expression of *Six2* at the earlier stages of SP development

points to the possibility that this gene might be critical during the stages of palatal shelf emergence (E12.5) and palatal shelf vertical outgrowth (E13.5). The rapid decline of *Six2* expression between E14.0 and E15.5 suggests that this gene may not be as critical during the later stages of palatogenesis.

In addition to demonstrating that that there are temporal changes in *Six2* expression levels during palatogenesis, my study also revealed regional differences in the spatial expression pattern of *Six2* in the developing SP. Fluorescence IHC demonstrated that Six2 protein was present in both the palate epithelium and palatal mesenchyme. Whereas Six2 protein is highly expressed in the nasal palatal epithelium (Fig. 10B and Fig. 20)), its expression is devoid from the oral palatal epithelium (Fig. 10D and Fig. 20). To confirm this finding, I utilized IHC double labelling of Six2 and E-cadherin, a known epithelial marker. The cells in the nasal palatal epithelium were positive for both Six2 and E-cadherin (Fig. 10J), while the cells lining the oral palatal epithelium were only positive for E-cadherin (Fig. 10L).

Interestingly, I observed an opposite pattern of Six2 protein expression in the palatal mesenchyme. Six2 protein was highly expressed in the oral half of the palatal mesenchyme, while reduced expression was observed in the nasal half of the palate mesenchyme (Fig. 11). This pattern of expression was consistent across the A-P axis of the palate and from E12.5 to E14.5 (Fig. 11A-C and Fig. 20). At E15.5 however, the pattern of Six2 protein expression in the mesenchyme seemed to be altered, with expression concentrated in the central portions of the fused palate (Fig. 11D).

Previous studies by different groups have also reported genes that exhibit differential expression patterns in the developing SP. Pax9, a gene that has been reported to play an important role in regulating epithelial-mesenchymal interactions during SP development is expressed in both the

mesenchyme and posterior palatal epithelium (Yu et al., 2013). Whereas Pax9 is expressed throughout the O-N axis of the palatal mesenchyme in the posterior region, its expression is lower in the oral half than in the nasal half of the palatal mesenchyme in the anterior region. Although the reason for this complimentary pattern of Six2 protein expression in the palate epithelium and mesenchyme is still unclear, it is possible that Six2 could be involved in the epithelial-mesenchymal cross-talk that drives palatal shelf outgrowth.

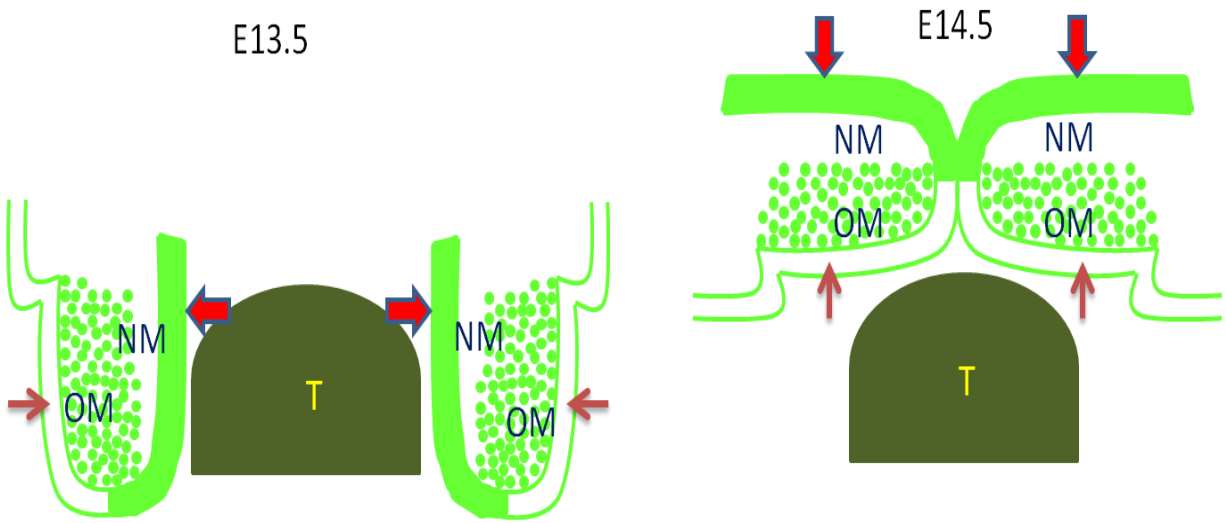


Figure 20: A schematic diagram illustrating the spatial expression pattern of Six2 protein in the developing palate at E13.5 and E14.5. Whereas there is expression of Six2 protein in the nasal palatal epithelium (block arrows) and the oral half of the palatal mesenchyme (OM), there is no expression in the oral palatal epithelium (stick arrows) and the nasal half of the palatal mesenchyme (NM), T-tongue.

7.2 Six2 protein exhibits an A-P differential expression pattern in the developing secondary palate.

The SP may be divided into three regions along the A-P axis namely; the anterior, medial and posterior palate. Various nomenclature systems have been used to designate the boundaries of the anterior, medial and posterior regions of the SP. When preparing coronal sections for this study, I followed the nomenclature of Smith et al., (2013) which defines tissue lying anterior to the first molar tooth bud as the anterior palate, tissue lying posterior to the first molar tooth bud as the posterior palate, and tissue in the plane of the first molar as the medial palate. Anterior, medial and posterior histological sections were taken from the same embryo. Using IHC analysis, I was able to demonstrate that Six2 protein exhibits an A-P differential expression profile in the developing SP. Although I observed a substantial amount of Six2 protein expression in the anterior regions of the palate mesenchyme, there appeared to be a gradual increase of Six2 protein expression towards the posterior regions of the palate mesenchyme. Also interestingly, it seems that Six2 protein is primarily expressed in the anterior nasal palatal epithelium and it progressively decreases along the A-P axis, such that little or no expression is seen in the most posterior nasal palatal epithelium (Fig. 12).

Previous studies have linked gene expression along the A-P axis to their functions in secondary palate development. For example, *Msx1* is a gene that is primarily expressed in the anterior portions of the SP, while *Mn1* is expressed mostly in the posterior regions of the palate. Whereas mice lacking either *Msx1* or *Mn1* exhibit a complete cleft palate, *Msx1* null mice exhibit specific cell proliferation defects in the anterior region, while *Mn1* null mice have growth deficits in only the middle and posterior regions of the palatal shelves (Zhang et al., 2002; Lin et al., 2008).

A plausible explanation for the disparity in the patterns of Six2 protein expression in the epithelium and mesenchyme of the SP along the A-P axis could be that in the epithelium, Six2 protein executes most of its functions in the anterior portions of the SP while in the palatal mesenchyme, most of its functions are performed in the posterior regions of the secondary palate.

7.3 *Six2* mRNA and protein are up-regulated in the absence of *Hoxa2*

A study by Kujetova et al., (2005) reported *Six2* to be an immediate downstream target of *Hoxa2*, a gene that has been found to play a direct role in mouse SP development (Smith et al., 2009). There have also been reports that the expression of *Six2* in the wild-type embryo is restricted to the first branchial arch mesenchyme, and that the loss of function of *Hoxa2* results in an expansion of the domain of *Six2* expression in the branchial arches and in the mesenchyme and leads to malformations of the middle and external ear. This implies that *Hoxa2* expression is sufficient to repress expression of *Six2* (Rijli et al., 1993; Kujetova et al., 2005). In my study, I sought to confirm if loss of *Hoxa2* function indeed alters the expression of *Six2* in the developing SP. My study utilized samples from palatal shelves of both wild-type and *Hoxa2* null embryos. Using qPCR and western blot analysis, I was able to demonstrate that *Six2* mRNA and protein respectively, were consistently up-regulated in the absence of *Hoxa2*, from the time of initial palatal shelf emergence at E12.5, through to the time of palatal shelf fusion at E15.5 (Fig. 13B, C). In addition, IHC analyses of anterior, medial and posterior palatal sections of both wild-type and *Hoxa2* null embryos across the stages of palatogenesis revealed more Six2 protein expression in the *Hoxa2* null embryos compared to their wild-type littermates (Fig. 13A).

Furthermore, *in vivo* cell counting analysis of Six2 protein positive cells in the nasal half of the palatal mesenchyme in wild-type and *Hoxa2* null littermates revealed an expanded domain of Six2 protein expression in the *Hoxa2* null palatal shelves. In the wild-type palatal shelves, Six2 protein expression was observed mainly in the oral half of the palatal mesenchyme with less expression observed in the nasal half of the palatal mesenchyme. In the *Hoxa2* null palatal shelves however, the domain of Six2 protein expression expanded from the oral half of the palatal mesenchyme to include the nasal half of the palatal mesenchyme where intense staining for Six2 protein was also observed (Figs. 13A, 14, 21). It is interesting to note that unlike the wild-type palatal shelves, the *Hoxa2* null palatal shelves failed to reorient themselves above the tongue but remained positioned vertically on either sides of the tongue (Fig.13A).

My findings therefore confirm that indeed, *Six2* is an immediate downstream target of *Hoxa2* and that *Hoxa2* expression alone is enough to repress the expression of *Six2*.

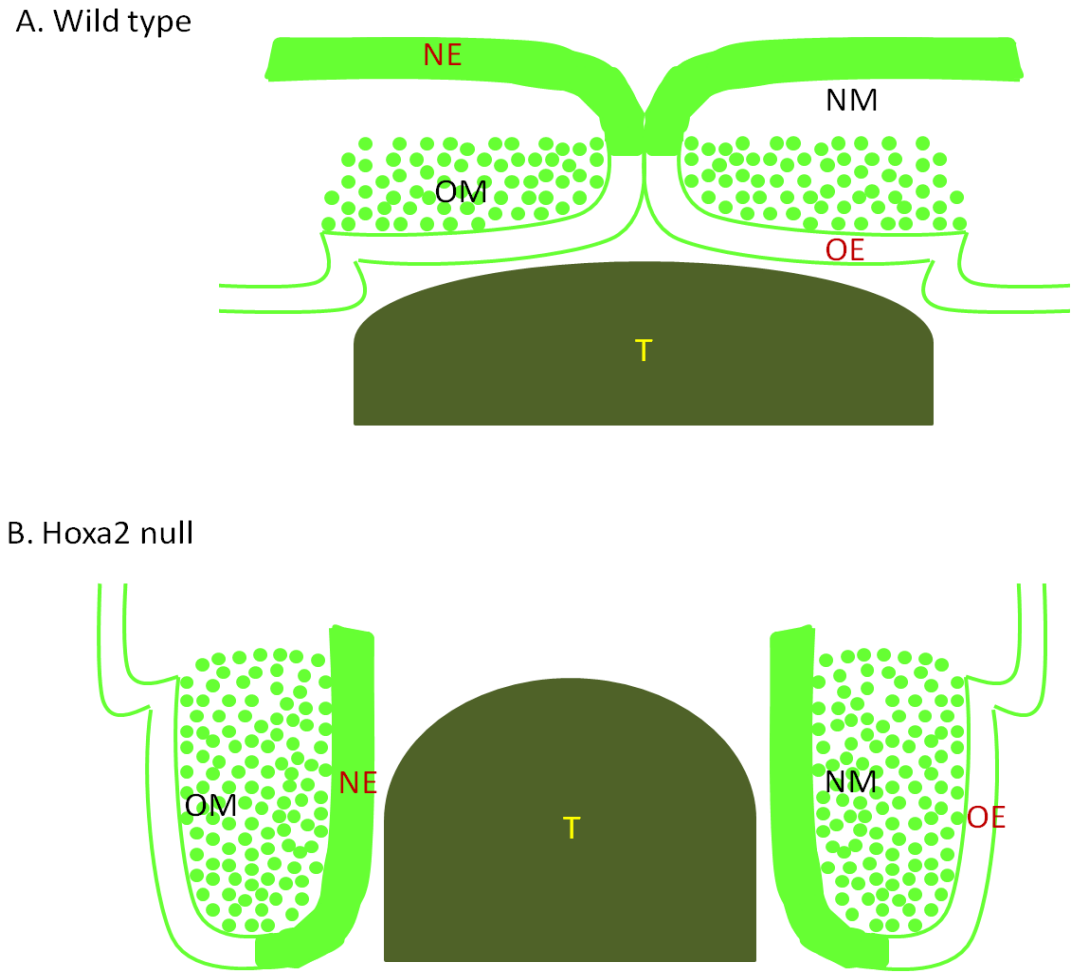


Figure 21: A schematic diagram demonstrating an expanded domain of Six2 protein expression in the mesenchyme of the *Hoxa2* null palatal shelf at E14.5.

A) In the wild-type palatal shelves, Six2 protein expression is observed mostly in the oral half of the palatal mesenchyme.

B) In the *Hoxa2* null palatal shelves however, the domain of Six2 protein expression in the mesenchyme expands to include the nasal half of the palatal mesenchyme. Also, unlike the wild-type palatal shelves, the *Hoxa2* null palatal shelves fail to re-orient themselves above the tongue. NE-nasal palatal epithelium, OE-oral palatal epithelium, NM-nasal half of palatal mesenchyme, OM-oral half of palatal mesenchyme, T-tongue.

7.4 *Six2* has an impact on palate mesenchymal cell proliferation during palatogenesis

7.4.1 There is increased cell proliferation and a higher percentage of cells co-expressing Six2 and Ki-67 in the nasal half of the posterior palatal mesenchyme of Hoxa2 null palatal shelves.

Six2 has been reported to be an immediate downstream target of *Hoxa2* and is up-regulated in *Hoxa2* null embryos (Santagati et al., 2005). In addition, a study by Smith et al., (2009) reported altered cell proliferation in *Hoxa2* null palatal shelves. My study therefore sought to investigate if the altered cell proliferation observed in the *Hoxa2* null palatal shelves is due to the up-regulation of *Six2*.

In order to investigate this potential role for *Six2* during palate mesenchymal cell proliferation, I performed IHC analyses on anterior, medial and posterior coronal palatal sections of both wild-type and *Hoxa2 null* embryos, using an antibody for the Ki-67 antigen which is a known marker of proliferating cells. I had initially wanted to use BrDU as opposed to Ki-67 but I encountered technical difficulties in double-labelling *Six2* and BrDU. I performed a cell counting analysis on whole palatal shelves and also the oral and nasal halves of the palatal mesenchyme. The cells counted included the total number of cells (Dapi positive cells), cells expressing Six2 protein (Six2 positive cells), actively proliferating cells (Ki-67 positive cells) and cells that were actively proliferating and co-expressing Six2 protein (cells positive for both Six2 and Ki-67). I chose to investigate this putative role of *Six2* at stage E13.5 because this is the stage at which *Six2* expression is highest at both the mRNA and protein level. Incidentally, this is also the stage when the palatal shelves start to grow vertically downwards on either sides of the tongue. Cell

counting analysis on whole palatal shelves after Ki-67 staining revealed a significant increase in cell proliferation in the *Hoxa2 null* palatal shelves compared to the wild-type palatal shelves in both the A-P and O-N axes of the palatal mesenchyme, with the highest percentage of proliferating cells observed in the nasal half of the posterior palatal mesenchyme of *Hoxa2 null* palatal shelves (Fig. 15). This finding is in agreement with that of Smith et al., (2009) who also observed increased cell proliferation in the *Hoxa2 null* palatal shelves.

Furthermore, I found that the percentage of actively proliferating cells that were co-expressing Six2 (Six2/Ki-67 double positive cells) was significantly increased in the *Hoxa2* null palatal shelves when compared to palatal shelves from wild-type embryos in both the A-P and O-N axes of the palatal mesenchyme, with the highest percentage of these double positive cells observed in the nasal half of the posterior palatal mesenchyme of *Hoxa2* null palatal shelves (Fig. 16).

As mentioned earlier, I observed Six2 protein expression exclusively in the nasal half of the palatal epithelium. In addition, I demonstrated that *Six2* mRNA and protein are up-regulated in the absence of *Hoxa2* and that Six2 protein expression is highest in the posterior regions of the secondary palate. These observations, coupled with the fact that I noticed increased cell proliferation and the highest percentage of actively proliferating cells that were co-expressing Six2 in the nasal half of the posterior palatal mesenchyme of *Hoxa2* null palatal shelves suggests a potential role for *Six2* during the palate epithelial-mesenchymal crosstalk that drives palate mesenchymal cell proliferation and palatal shelf outgrowth.

7.4.2 In vitro *Six2* siRNA knockdown restored cell proliferation and *Cyclin D1* expression in Hoxa2 null MEPM cell cultures to wild-type levels

In order to further investigate the role of *Six2* in palate mesenchymal cell proliferation, I performed *in vitro Six2* siRNA knockdown in MEPM primary cells obtained from palatal shelves of both wild-type and *Hoxa2* null mouse embryos. The palatal shelves were harvested at E13.5 when *Six2* expression is at its peak. I mentioned earlier that my study has confirmed previous reports that there is increased palate mesenchymal cell proliferation in *Hoxa2* null palatal shelves compared to wild-type palatal shelves. In addition, my study reveals enhanced *Six2* expression in the absence of *Hoxa2*. It was therefore interesting to discover that *in vitro Six2* siRNA knockdown restored palate mesenchymal cell proliferation in the *Hoxa2* null MEPM cell cultures to wild-type levels (Fig. 18A). This finding points to the possibility that the increased cell proliferation observed after knocking out *Hoxa2*, could in part, be due to enhanced *Six2* expression. Zhang et al., (2002) and Rice et al., (2004) found that exogenous application of *Shh* induced a mitogenic response in palatal explants cultures mediated in part by the cell cycle regulators *Cyclin D1* and *Cyclin D2*. In this study, I found that *in vitro Six2* siRNA knockdown also restored *Cyclin D1* expression in the *Hoxa2* null MEPM cell cultures to wild-type levels (Fig. 18B). This implies that the cell cycle regulator *Cyclin D1* is acting downstream of *Six2* in the palate mesenchyme. This finding re-affirmed my initial suspicions that *Six2* might play a crucial role in palate mesenchymal cell proliferation.

My study further investigated if *in vitro Six2* siRNA knock down had an effect on the expression of *Msx1*, *Bmp4*, *Barx1* and *Ptx1*, some of the genes involved in palate mesenchymal cell signalling during SP development (Smith

et al., 2009). *In vitro Six2* siRNA knockdown however, did not alter the expression of any of the above mentioned genes (Fig. 19). A possible explanation for this observation could be that these genes are acting upstream of *Six2* in the mesenchyme during SP development. Collectively, the ability of actively proliferating palate mesenchymal cells to co-express Six2 protein and the restoration of palate mesenchymal cell proliferation and *Cyclin D1* expression in *Hoxa2* null MEPM cell cultures to wild-type levels points to the possibility that *Six2* might have a role to play in palate mesenchymal cell proliferation. In addition, this finding has confirmed the cell cycle regulator *Cyclin D1* as a putative downstream target of *Six2* in the palate mesenchyme.

8. CONCLUSION

Although *Six2* has been widely studied in mammalian embryonic development, its precise role during mammalian secondary palate development has never been reported. My study reveals for the first time that *Six2* is expressed during all stages of secondary palate development with peak expression occurring at E13.5 at both the mRNA and protein level. My results also show that Six2 protein is expressed in the nasal palatal epithelium but is completely absent from the oral palatal epithelium. In addition, I observed increased staining for Six2 protein in the oral half of the palatal mesenchyme and reduced staining for Six2 protein in the nasal half of the palatal mesenchyme. My study also demonstrates that Six2 protein exhibits an A-P differential expression pattern in the developing SP. Six2 protein expression in the palatal mesenchyme increases in the anterior to posterior direction with the most expression observed in the posterior regions of the palate. On the contrary, Six2 protein expression in the nasal palatal epithelium seems to decrease as one moves from the anterior regions of the palate to the posterior regions of the palate.

Furthermore, I show that *Six2* is up-regulated in the absence of *Hoxa2* at both the mRNA and protein level, consistent with earlier reports that *Hoxa2* binds to the *Six2* promoter *in vivo* to repress its expression. I also successfully demonstrated that actively proliferating palate mesenchymal cells were co-expressing Six2 protein, indicating a potential role for *Six2* during palate mesenchymal cell proliferation. This finding was further strengthened by *in vitro* studies on primary MEPM cell cultures which showed that *in vitro Six2* siRNA knockdown restored cell proliferation and *Cyclin D1* expression in the *Hoxa2* null MEPM cell cultures to wild type levels.

Collectively, my data reveals a novel expression profile for *Six2* in the developing murine SP and suggests a possible role it might play during the epithelial-mesenchymal crosstalk that drives palatal shelf cell proliferation and outgrowth.

9. FUTURE WORK CONSIDERATIONS

My study sought to find out if *Six2* is expressed in the developing murine SP, and if it is, what role it might be playing during SP development. Although I can confidently state that all the experimental objectives of my thesis project were met, my study raises at least as many questions as it has answered. My data reveals a detailed expression analysis of *Six2* in the developing SP. Using qPCR and Western blot analyses, I demonstrate that *Six2* is expressed across all stages of SP development, while IHC reveals the spatial expression profile of this gene product in both the epithelium and mesenchyme of the developing palate. However, studies on precise *Six2* mRNA localization need to be conducted so as to consolidate the findings of this study in regard to expression analysis. This can be conducted by *in situ* hybridization studies with use of palatal tissue sections or whole mount *in situ* hybridization.
I have also shown that Six2 is mostly expressed during the earlier stages of SP development (E12.5-E13.5), suggesting that it might perform most of its functions at this earlier stage. In this study, *in vitro* cell proliferation analysis using MEPM cells showed that siRNA knockdown of *Six2* in *Hoxa2* null cells restored cell proliferation and *Cyclin D1* expression to wild type levels. This finding could be strengthened by using the palatal shelves of a *Six2* knock out mouse model instead of MEPM cell cultures. It would also be interesting to find out if the *Six2* knockout mouse has any defects in palate morphology. In addition, *in vivo* cell counting analysis using a cell proliferation marker such as Ki-67 or Brdu could be performed on coronal palate sections to directly compare cell proliferation in the *Six2* null and wild-type mice. Also, a *Six2/Hoxa2* double knock out mouse model could shed more light on the restoration to wild-type levels of cell proliferation and expression of cell cycle regulators such as *Cyclin D1* and *Cyclin D2*. A study can also be designed to

investigate if epithelial specific inactivation of *Six2* (by silencing *Six2* in palatal epithelial cell cultures) could have an effect on palate mesenchymal cell proliferation.

Furthermore, the palatal cell signalling pathways that *Six2* might be involved in are yet to be studied. The *Shh* signalling pathway is of particular interest because this gene is expressed in the oral palatal epithelium while *Six2* is also highly expressed in the oral half of the underlying palate mesenchyme. It is therefore possible that epithelial-mesenchymal interactions exist between *Shh* in the epithelium and *Six2* in the underlying mesenchyme. In addition, previous studies have revealed that the expression of *Osr1* and *Osr2*, which are some of the genes that are exclusively expressed in the oral half of the palatal mesenchyme is dependent on *Shh* signalling (Fig. 22). It would therefore be interesting to investigate if a linkage exists between *Shh*, *Six2*, *Osr1* and *Osr2*.

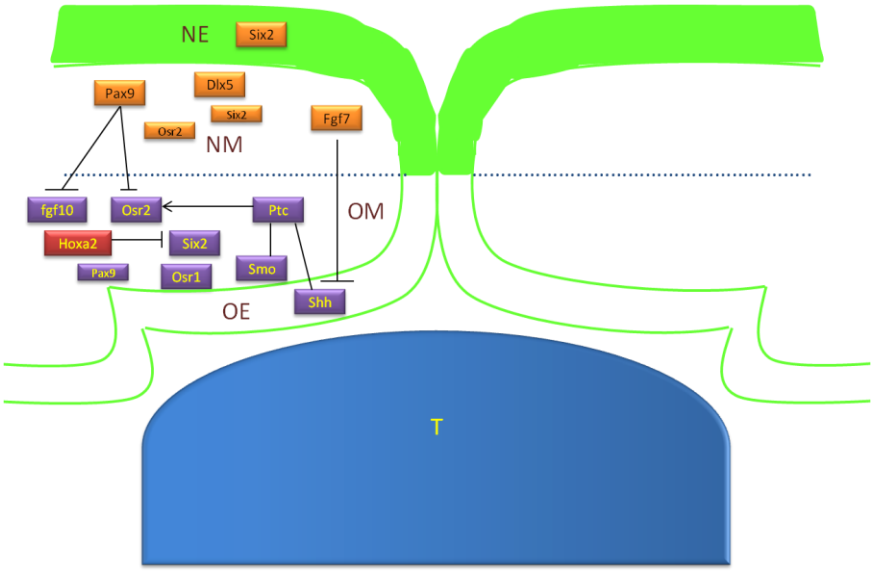


Figure 22: A schematic illustration of the pathways involved in O-N patterning during palatal shelf out growth.

Whereas *Pax9* is expressed across the O-N axis of the posterior palatal mesenchyme, it is expressed at higher levels in the nasal half of the palatal mesenchyme than in the oral half of the palatal mesenchyme in the anterior regions of the palate. In addition, *Pax9* has been reported to have inhibitory effects on both *Osr2* and *Fgf10* in the palate mesenchyme. *Fgf10* is exclusively expressed in the oral half of the palatal mesenchyme. *Six2* also exhibits a graded pattern of expression in the palate mesenchyme with higher expression in the oral half of the palate mesenchyme than in the nasal half of the palate mesenchyme. *Six2* is highly expressed in the nasal palate epithelium but is devoid from the oral palatal epithelium. Hoxa2 has been reported to have inhibitory effects on *Six2* expression. Whereas *Osr1* expression is limited to the oral half of the palate mesenchyme, *Osr2* is expressed at high levels in the oral half of the palatal mesenchyme than in the nasal half. Expression of both *Osr1* and *Osr2* is dependent on *Shh* signalling. Also, a pathway involving *Dlx5-Fgf7* signalling acts as a repressor of *Shh* signalling and controls outgrowth along the ON axis. The dotted line denotes the O-N boundary of the palatal mesenchyme. Nasal palatal epithelium (NE), Nasal half of the palatal mesenchyme (NM), Oral palatal epithelium (OE), Oral half of the palatal mesenchyme (OM), Tongue (T). Arrows indicate inductive relationships, solid lines represent direct physical interaction while blunt arrows indicate inhibitory effects. Purple boxes indicate expression in the oral half of the palate mesenchyme while orange boxes indicate expression in the nasal half of the palate mesenchyme. The size of the boxes is directly proportional to the extent of expression in the epithelium and the oral or nasal half of the palate mesenchyme.

Pax9 has been reported to have an inhibitory effect on the expression of both *Fgf10* and *Osr2*. *Pax9* mRNA has been reported to be preferentially expressed in the nasal half of the palatal mesenchyme in the anterior palate. Incidentally, my study shows that *Six2* is present only in the nasal palatal epithelium and that there are more actively proliferating cells that co-express *Six2* in the nasal half of the palate mesenchyme. Additional studies therefore can be conducted to reveal the relationship between *Six2, Pax9, Osr2* and *Fgf10* in the nasal half of the palate mesenchyme. *Fgf7 and Dlx5* are the other genes that have been found to be expressed spatially and temporally in the developing palate. Additionally, tissue specific inactivation of *Six2* in either the epithelium or mesenchyme of the palate can be used to find out if *Six2* is acting upstream of any of these genes to regulate their expression. This study also reported that the knock down of *Six2* in MEPM cells did not have an effect on the expression of *Bmp4*, *Msx1, Barx1* and *Ptx1*. This could be because these genes might be acting up stream of *Six2*. Further investigations using Chromatin immunoprecipitation (ChIP) analysis can be performed to confirm whether these genes are indeed acting upstream to regulate *Six2* expression.

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11. APPENDIX

In vivo cell counting analysis

Table 1: N of 1

Dapi positive cells (total cell number)						
	Anterior		**Medial**		**Posterior**	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
Wild type	134	148	320	144	256	283
***Hoxa2* null**	144	170	182	206	245	266
Ki-67 positive cells						
Wild type	49	72	108	154	157	261
***Hoxa2* null**	90	120	124	173	170	228
Six2/Ki-67 double positive cells						
Wild type	22	39	56	92	130	140
***Hoxa2* null**	34	43	72	105	127	91
Six2 positive cells						
Wild type	92	84	256	159	182	127
***Hoxa2* null**	138	109	179	182	232	205

Table 2: N of 2

Dapi positive cells (total cell number)						
	Anterior		**Medial**		**Posterior**	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
Wild type	223	262	247	250	254	291
***Hoxa2* null**	236	227	202	181	247	277
Ki-67 positive cells						
Wild type	74	90	75	100	63	97
***Hoxa2* null**	107	126	98	154	168	193
Six2/Ki-67 double positive cells						
Wild type	53	50	54	65	56	59
***Hoxa2* null**	74	77	77	112	125	107
Six2 positive cells						
Wild type	170	145	180	154	189	171
***Hoxa2* null**	200	170	196	168	241	236

Table 3: N of 3

Dapi positive cells (total cell number)						
	Anterior		**Medial**		**Posterior**	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
Wild type	164	184	172	194	232	291
***Hoxa2* null**	142	147	193	240	250	211
Ki-67 positive cells						
Wild type	82	95	95	101	102	118
***Hoxa2* null**	83	92	82	140	120	136
Six2/Ki-67 double positive cells						
Wild type	34	42	77	78	55	70
***Hoxa2* null**	35	26	65	87	82	104
Six2 positive cells						
Wild type	96	82	129	102	145	112
***Hoxa2* null**	121	114	181	179	244	198

Table 4: N of 4

Dapi positive cells (total cell number)						
	Anterior		**Medial**		**Posterior**	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
Wild type	220	247	193	240	203	200
***Hoxa2* null**	88	123	145	160	225	208
Ki-67 positive cells						
Wild type	41	90	128	137	81	91
***Hoxa2* null**	59	66	72	111	124	121
Six2/Ki-67 double positive cells						
Wild type	46	44	104	95	51	58
***Hoxa2* null**	47	46	68	95	95	90
Six2 positive cells						
Wild type	165	148	144	135	155	148
***Hoxa2* null**	83	94	139	132	220	178

Table 5: Ki-67 positive cells as a percentage of Dapi positive (total) cells in the whole palate.

N	Anterior		Medial		Posterior	
	Wild type	***Hoxa2* null**	**Wild type**	***Hoxa2* null**	**Wild type**	***Hoxa2* null**
1	42.91%	66.88%	38.78%	76.55%	47.20%	93.54%
2	33.81%	50.32%	35.21%	65.80%	43.89%	68.89%
3	43.25%	60.55%	53.55%	51.27%	42.45%	70.53%
4	28.05%	59.24%	37.23%	60.00%	58.56%	72.65%

Table 6: Ki-67 positive cells as a percentage of Dapi positive (total) cells in the Wild type palate; Oral vs. Nasal.

N	Anterior		Medial		Posterior	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
1	35.57%	48.65%	40.37%	52.49%	61.33%	76.87%
2	33.18%	34.35%	30.36%	40.00%	44.98%	58.99%
3	46.64%	51.63%	47.22%	52.06%	43.97%	63.45%
4	18.64%	36.44%	45.32%	54.34%	39.90%	49.19%

Table 7: Ki-67 positive cells as a percentage of Dapi positive (total) cells in the *Hoxa2* null palate; Oral vs. Nasal.

N	Anterior		Medial		Posterior	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
1	43.78%	70.59%	63.13%	83.98%	69.39%	85.71%
2	45.34%	55.10%	48.51%	75.67%	68.02%	88.78%
3	58.45%	62.59%	42.49%	58.33%	48.00%	71.09%
4	47.98%	53.66%	49.66%	69.38%	55.10%	72.02%

Table 8: Six2 positive cells as a percentage of Dapi positive (total) cells in the whole palate.

N	Anterior		Medial		Posterior	
	Wild type	***Hoxa2* null**	**Wild type**	***Hoxa2* null**	**Wild type**	***Hoxa2* null**
1	62.77%	76.23%	73.58%	81.45%	57.33%	96.28%
2	64.99%	79.91%	67.20%	89.49%	76.06%	98.87%
3	51.15%	81.31%	63.11%	87.25%	69.67%	95.88%
4	67.02%	74.89%	67.55%	84.61%	78.79%	78.99%

Table 9: Six2/Ki-67 double positive cells as a percentage of Six2 positive cells in the whole palate.

N	Anterior		Medial		Posterior	
	Wild type	***Hoxa2* null**	**Wild type**	***Hoxa2* null**	**Wild type**	***Hoxa2* null**
1	27.79%	46.41%	35.66%	49.03%	52.51%	67.42%
2	32.70%	40.81%	35.63%	51.92%	52.32%	61.99%
3	42.70%	25.98%	48.98%	42.22%	48.64%	64.76%
4	28.75%	52.54%	42.64%	60.15%	47.35%	46.48%

Table 10: Six2/Ki-67 double positive cells as a percentage of Six2 positive cells in the Wild type palate; Oral vs Nasal.

N	Anterior		Medial		Posterior	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
1	23.91%	31.24%	21.88%	57.86%	63.21%	71.41%
2	31.18%	34.48%	30.00%	42.21%	29.63%	34.50%
3	36.96%	27.86%	35.91%	44.70%	37.93%	62.50%
4	27.88%	34.13%	43.22%	42.78%	32.90%	39.19%

Table 11: Six2/Ki-67 double positive cells as a percentage of Six2 positive cells in the *Hoxa2* null palate; Oral vs Nasal.

N	Anterior		Medial		Posterior	
	Oral	**Nasal**	**Oral**	**Nasal**	**Oral**	**Nasal**
1	38.41%	39.45%	40.22%	57.69%	54.74%	61.75%
2	37.00%	45.29%	39.29%	61.54%	51.87%	64.55%
3	28.93%	41.42%	47.32%	48.60%	45.90%	69.95%
4	56.63%	39.79%	48.92%	65.78%	43.18%	50.56%

Absolute quantitative expression of palatal *Six2* mRNA by droplet digital PCR

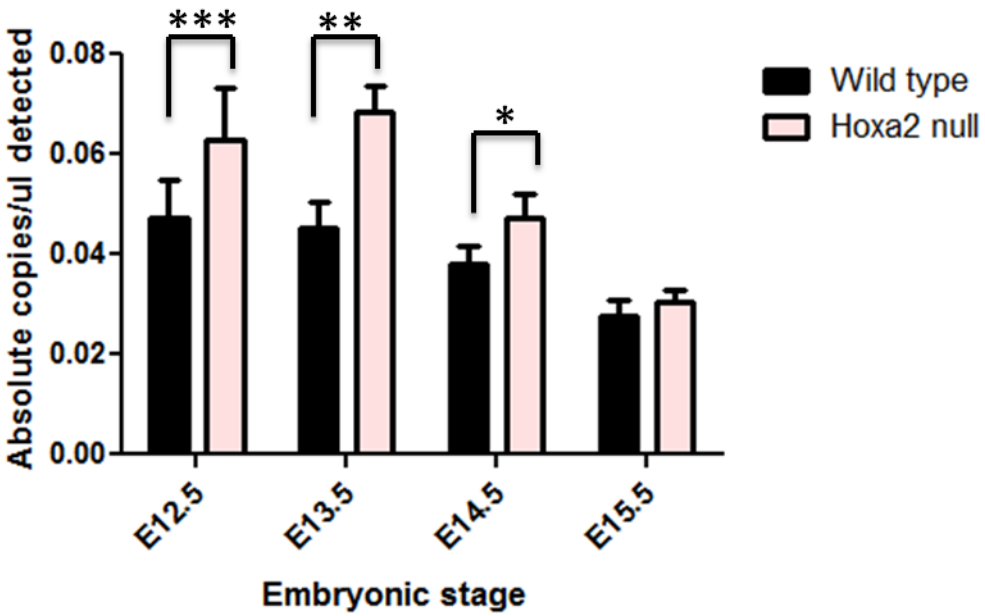

Figure 1: Six2 mRNA expression by ddPCR. Absolute quantitative expression of *Six2 mRNA* during murine palatogenesis in wild type and *Hoxa2* null mice. In the wild type mice, *Six2* mRNA expression is comparably high at E12.5 and E13.5 and reduces at E14.5 before decreasing further at E15.5. In the *Hoxa2* null mice, expression is enhanced across all stages of palatogenesis. In the absence of *Hoxa2, Six2* mRNA is high at E12.5 and increases to a peak level at E13.5. This expression dramatically reduces at E14.5 before decreasing further at E15.5. Bars represent mean ± SEM, n=5, *p<0.05, **p<0.01.

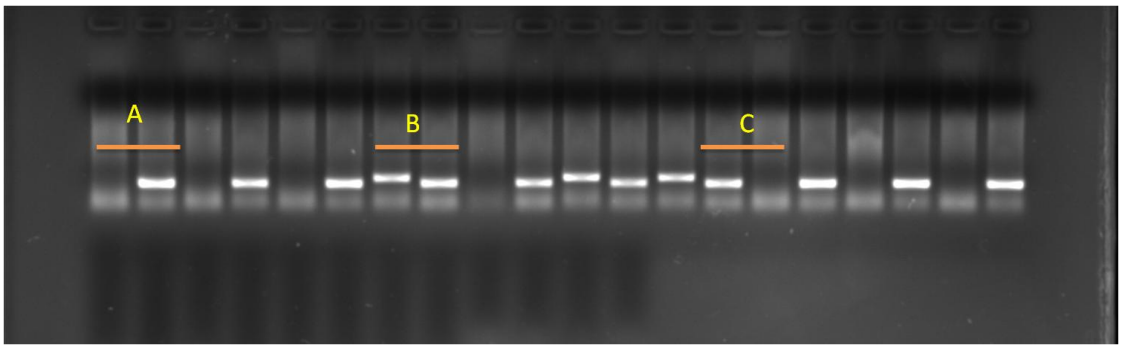

Figure 2. Sample of the Genotyping Results of *Hoxa2* Transgenic Mice. A gel (using Red Safe) of the PCR analysis used in the genotyping of *Hoxa2* transgenic mice. The presence of a band with the wild-type primers only (A) confirms that the animal is wild-type. If a band is present only with the neomycin primers (C) this confirms the embryo is *Hoxa2* null. Presence of both bands indicates that the animal is a heterozygote (B).

Table 12: Primers used for qPCR analysis of *Msx1, Bmp4, Barx1 and Ptx1*.

	Forward primer	**Reverse primer**
Msx1	CTCGGTGTCAAAGTGGAGG	GCTGAAGGGCAGGAGTGAA
Barx1	ATTGCGAGGACTGAGCGT	GACACCTGGGATTGGCTTC
Bmp4	AGGAAGGAGTAGATGTGAGAG	AGGGACGGAGACCAGATAC
Ptx1	ACCCGAGCCTTAGAAACC	GACCTGTTGGGGACAAGA
Six2	GCCAAGGAAAGGGAGAACAGC	GCGTCTTCTCATCCTCGGAAC

A: Six2- 314bp

B: Msx1- 153bp

C: Barx1- 175bp

D: Bmp4- 158bp

E: Ptx1- 214bp

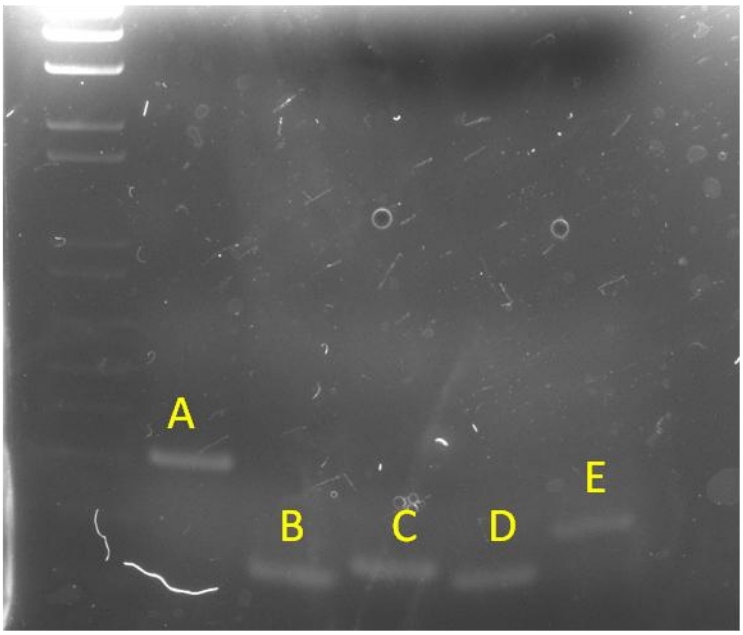

Figure 3: An agarose gel showing the integrity of the primers that were used for PCR genotyping analysis. All bands were single with no formation of primer- dimmers.

Table of Reagent catalogue numbers

Reagent name	**Company name**	**Calatogue number**
Six2 Taqman probes	Applied Biosystems	Mm03003557_S1
Six2 antibody	Protein Tech	11562-A-P
Msx1 Sybr green primers	Invitrogen	10336022
Bmp4 Sybr green primers	Invitrogen	10336022
Barx1 Sybr green primers	Invitrogen	10336022
Ptx1 Sybr green primers	Invitrogen	10336022
Lipofectamine 3000	Invitrogen	1559381
Six2 silencer select siRNA	Life Technologies	4390771
E-cadherin antibody	Sigma	U 3254
Ki-67 antibody	Affymetrix e-bioscience	13-5698-82

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